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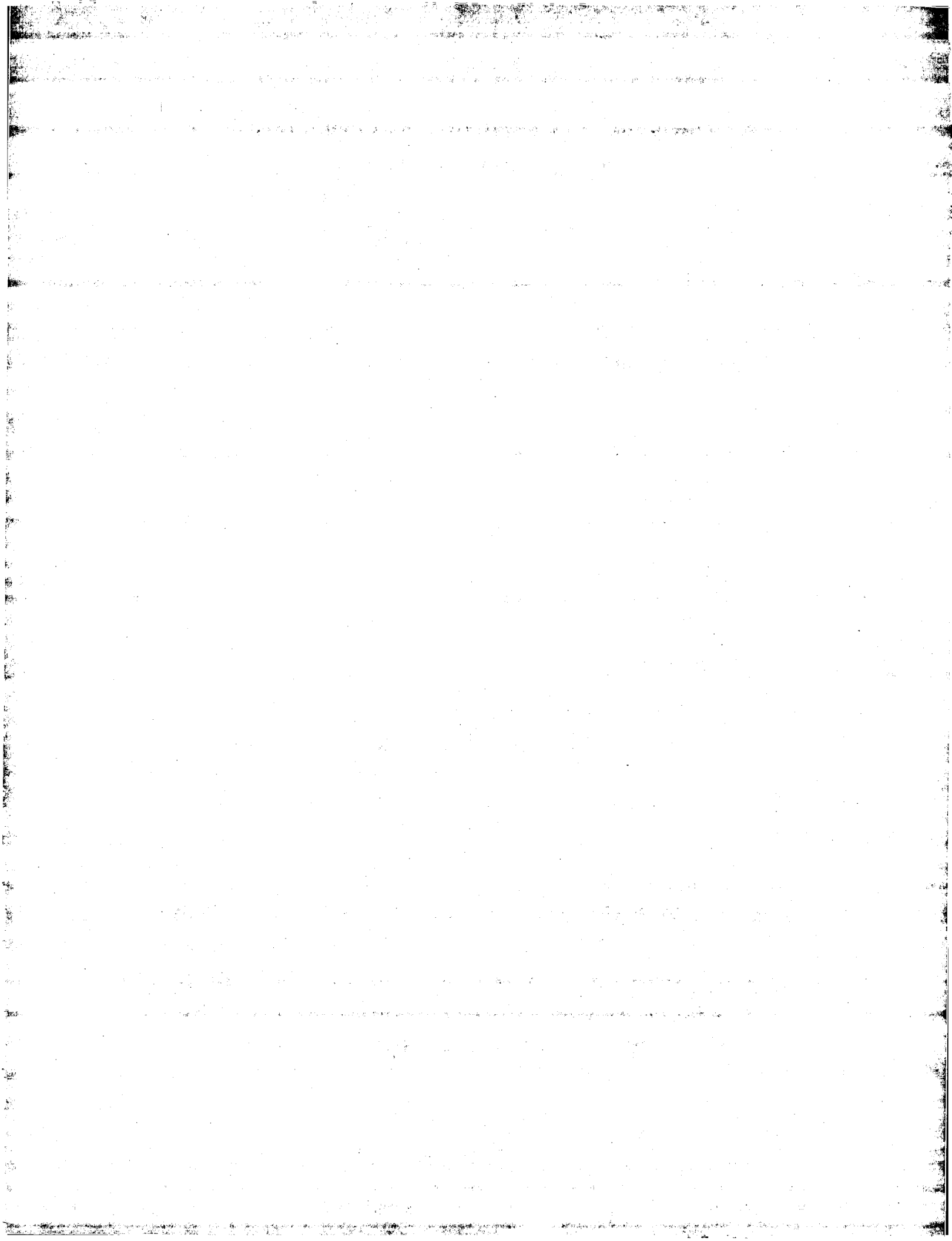
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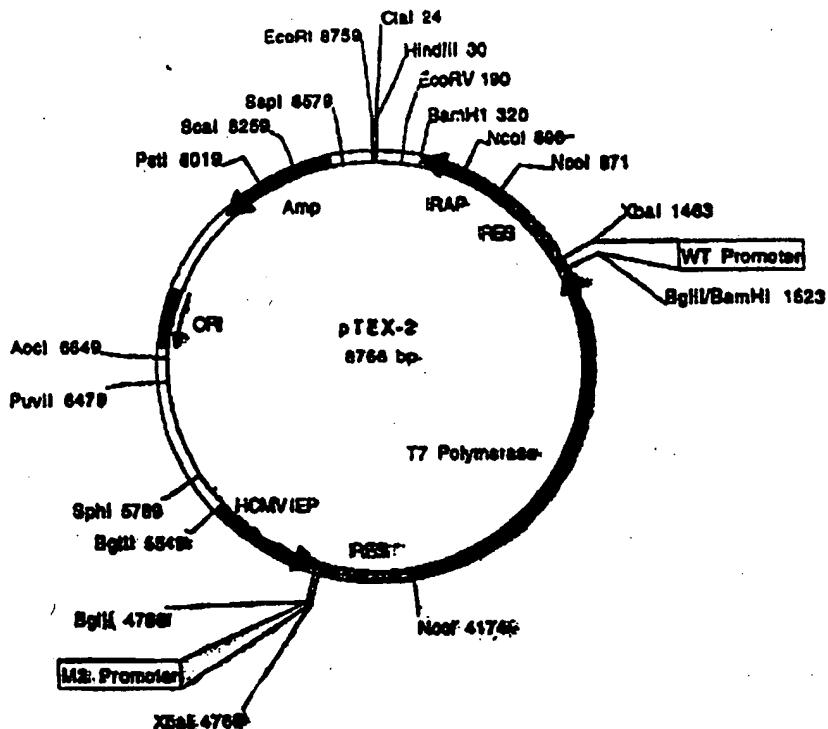
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## (57) Abstract

The present invention discloses novel prokaryotic RNA polymerase autogenes which can be used in eukaryotic cytoplasmic expression systems.



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**A CYTOPLASMIC GENE EXPRESSION SYSTEM WHICH UTILIZES  
A PROKARYOTIC RNA POLYMERASE AUTOGENE**

This application claims the benefit of United States Provisional Application 60/016,304 filed April 26, 1996, the contents of which are incorporated herein by reference.

**FIELD OF INVENTION**

The present invention relates to the expression of nucleic acid sequences in eukaryotic cells. More specifically, the invention relates to a cytoplasmic expression system which utilizes prokaryotic RNA polymerase autogenes to facilitate the cytoplasmic expression of DNA in cells.

**BACKGROUND OF INVENTION**

To date, a number of expression systems have been utilized to express genes in eukaryotic cells both in vitro and in vivo. However, since many of these systems consist of a DNA expression vector which contains the gene of interest under control of a eukaryotic promoter, these expression systems require entry of the DNA vector into the host cell nuclei where the transcriptional machinery for the eukaryotic promoter resides. Unfortunately, only a very small percentage of the DNA taken up by eukaryotic cells is transported to the nucleus; therefore, expression of genes from such nuclear expression systems can be limited. Accordingly, expression vectors which contain the gene of interest under the control of prokaryotic promoters have been designed to drive expression in the cytoplasm. This is beneficial since cytoplasmic uptake of DNA by eukaryotic cells is more efficient and prokaryotic RNA polymerases reside only in the cytoplasm of eukaryotic cells, due to the absence of a nuclear localization signal

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in the molecules. However, even such cytoplasmic expression systems have drawbacks since high, continuous levels of expression of DNA in cells via such systems is dependent on a continuous supply of prokaryotic RNA polymerase in the cytoplasm of the cells. Thus, for example, T7 expression vectors must be codelivered to cells with an exogenous supply of T7 RNA polymerase or utilized in cell lines which express T7 RNA polymerase. Unfortunately the former approach is both costly and potentially toxic to cells while the latter approach places a major limitation on the use of T7 expression vectors in eukaryotic cells since the T7 RNA polymerase gene is a bacterial gene not expressed endogenously in eukaryotic cells. For these reasons, investigators have attempted to design T7 RNA polymerase autogenes which would function as a self-initiating and self-amplifying source of T7 RNA polymerase in the cytoplasm of eukaryotic cells.

Gao et al. ((1993) Nucleic Acids Res. 21: 2867-2872) described a T7 autogene designated pT7AUTO2C, that successfully supported cytoplasmic expression in eukaryotic cells of a reporter gene driven by the bacteriophage T7 promoter. However, this autogene was able to produce T7 RNA polymerase only when codelivered to cells with an amount of exogenous T7 RNA polymerase sufficient to initiate the autocatalytic production of T7 RNA polymerase from the T7 promoter of pT7AUTO2C. Thus, the pT7AUTO2C autogene was not truly a self-generating source of T7 polymerase in that it required delivery of exogenous T7 polymerase to start the autocatalytic cycle.

Deng et al. (1993) Gene , 143:245-249) refer to an autogene plasmid which contains the T7 promoter, the encephalomyocarditis internal ribosomal entry sequence (EMC IRES) and the T7 RNA polymerase gene. In vitro transcripts of this autogene alone, or in combination with the autogene plasmid DNA, were reported to support foreign

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gene expression in vitro. However, foreign gene expression supported by autogene plasmid DNA alone was only reported for a cell line which stably expressed low levels of T7 RNA polymerase sufficient to apparently initiate transcription of the T7 RNA polymerase gene from the T7 promoter of the autogene plasmid DNA.

WO 94/26911 refers to a construct which contains a T7 RNA polymerase gene, an EMC IRES and a T7 promoter. Incubation of this construct with T7 RNA polymerase is reported to result in binding of the T7 RNA polymerase to the construct prior to the introduction of the construct into cells thereby permitting the T7 RNA polymerase: construct complex to serve as a self-initiating and self-amplifying source of T7 RNA polymerase upon entry into the cytoplasm of cells. However, there still exists a need for a T7 RNA polymerase autogene that can yield continuous, high level expression of T7 RNA polymerase in eukaryotic cells without the need for exogenous T7 polymerase enzyme to initiate the amplification process. Such autogenes would be useful for both in vitro and in vivo protein expression, including use in gene therapy. Gene therapy has become one of the fastest developing fields of biomedicine in recent years (Anderson, W.F. (1992) Science, 256: 808-813).

#### SUMMARY OF INVENTION

This invention relates to a cytoplasmic gene expression system which utilizes a self-initiating, self-amplifying prokaryotic RNA polymerase autogene.

The prokaryotic RNA polymerase autogene of the invention is a DNA construct which includes at a minimum, the following elements operatively linked in 5' to 3' order: a eukaryotic promoter, a cognate promoter of the prokaryotic RNA polymerase and a nucleic acid sequence encoding the a prokaryotic RNA polymerase. In another

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embodiment, the cognate promoter in the autogene construct may be separated from the RNA polymerase gene by an internal ribosome entry site sequence (IRES).

The invention further relates to the use of this autogene in cytoplasmic expression systems in vitro and in vivo to express target sequences.

In one embodiment, the cytoplasmic expression system is a "dual sequence cytoplasmic expression system" which contains a first sequence comprising the prokaryotic RNA polymerase autogene and a second sequence comprising the cognate RNA polymerase promoter operatively linked to a target sequence.

In another embodiment, the cytoplasmic expression system may be a "single sequence cytoplasmic expression system" which consists of a single nucleic acid sequence containing in 5' to 3' order: a eukaryotic promoter, a cognate promoter of the prokaryotic RNA polymerase, a nucleic acid sequence encoding the RNA polymerase, a second copy of the cognate promoter and a target sequence. In another embodiment, the cognate promoter is separated from the target sequence in the dual and single sequence systems by an IRES.

The invention further provides pharmaceutical compositions comprising either the dual or single sequence cytoplasmic expression systems. In addition, depending on the molecule encoded by the target sequence contained in the pharmaceutical compositions, the compositions of the invention may be administered prophylactically and/or therapeutically.

The invention also relates to a kit comprising the prokaryotic RNA polymerase autogene alone, in combination with a second sequence containing the cognate RNA polymerase promoter operatively linked to a target sequence as a dual sequence cytoplasmic expression system, or as a single sequence in combination with a second copy of the cognate RNA polymerase promoter operatively linked



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to a target sequence.

The invention also provides cell lines stably transformed with the prokaryotic RNA polymerase autogene of the invention.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows maps of the linear DNA fragments and conventional plasmids used in the transfection studies. Sequences of primers used to generate the linear PCR fragments are also shown. The abbreviations for the DNA sequences are as follows: pT7-CAT: T7-CAT plasmid; fT7-CAT: T7-CAT PCR fragment; pUCCMV-CAT: CMV-CAT plasmid; fCMV-CAT: CMV-CAT PCR fragment; pCMV/T7-T7pol: the T7 autogene plasmid where the T7 RNA polymerase gene is under the control of both CMV and T7 promoters; pT7: T7 promoter; tT7: T7 terminator; T7pol: T7 RNA polymerase gene; IRES: internal ribosome entry site sequence of encephalomyocarditis virus.

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Figure 2 shows plasmid maps for pcDNA3, pAR3126 and pCMV/T7-T7pol. A HindIII/BamHI fragment from pAR3126 containing the cDNA of T7 RNA polymerase was inserted into the corresponding sites of the pcDNA3 plasmid vector to generate the pCMV/T7-T7pol autogene.

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Figure 3 shows a plasmid map for T7 RNA polymerase autogene designated PAUTOM2-C which comprises in 5'-3' order: a CMV immediate early promoter; a mutant T7 promoter (m2 Promoter) an Encv IRES, a T7 RNA polymerase gene and a T7 terminator.

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Figure 4 shows a plasmid map for a T7 RNA polymerase autogene designated PT7-H1-AUTOD which comprises in 5' to 3' order: a CMV immediate early promoter, a T7 promoter fused to the lac operator, an Encv

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IRES, a T7 RNA polymerase gene and a T7 terminator.

Figure 5 shows a schematic of a single sequence cytoplasmic expression system which contains in a single nucleic acid sequence the following elements in 5' to 3' order: a CMV promoter, a T7 promoter, a T7 RNA polymerase gene, a second copy of the T7 promoter, an EMCV IRES and a CAT reporter gene.

Figure 6 shows a schematic of a single sequence cytoplasmic expression system designated pTex 2 which contains in a single nucleic acid sequence, the following elements in 5' to 3' order: a CMV immediate early promoter, a mutant T7 promoter (m2), an IRES, T7 RNA polymerase gene, a wild-type T7 promoter (T7 WT), an IRES and an IRAP reporter gene.

Figure 7 shows CAT activity as a function of pT7-CAT and fT7-CAT DNA concentration. Various amounts of pT7-CAT or fT7-CAT DNA, complexed with DC-chol:DOPE liposomes at a ratio of 10 nmol lipid/ $\mu$ g, were used to transfect 293-T7 cells for 4 hours. CAT assays were performed 48 hours following the transfection.

Figure 8 shows CAT expression as a function of time. 0.27 pmol of pT7-CAT or fT7-CAT DNA was delivered to 293-T7 cells by DC-chol:DOPE liposomes as described in Figure 4. Cells were collected daily for 9 days and split 1:1 once on day 4 post-transfection when they were 100% confluent.

Figure 9 shows CAT activity 48 hours following cotransfection of normal 293 cells for 4 hours with a mixture of 0.27 pmol pT7-CAT or fT7CAT and varying mole amounts of pCMV/T7-T7pol autogene complexed with DC-chol:DOPE liposomes (10 nmol lipid/ $\mu$ g DNA).

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Figure 10 shows CAT activity in normal 293 cells as a function of pT7-CAT and fT7-CAT DNA concentration. 293 cells were transfected for 4 hours with various amounts of pT7-CAT or fT7-CAT codelivered with pCMV/T7-T7pol (5:1 mol/mol of pT7-CAT or fT7-CAT to pCMV/T7-T7pol) by DC-chol:DOPE liposomes (10 nmol lipid/ $\mu$ g DNA). CAT assays were performed 48 hours following transfection.

Figures 11A and 11B show CAT expression (Figure 11A) and toxicity (Figure 11B) as a function of time in 293 cells transfected for four hours with 0.27 pmol pT7-CAT or fT7-CAT and 54.11 fmol pCMV/T7-T7pol autogene complexed with DC-Chol: DOPE liposomes (10 nmol lipid/ $\mu$ g DNA). In Figure 11A, CAT activity was determined 48 hours after transfection and in Figure 11B, toxicity was measured as protein recovered in transfected cells 48 hours after transfection.

Figures 12A and 12B show transfection efficiency (Figure 12A) and toxicity (Figure 12B) of 293 cells co-transfected for four hours with pT7-CAT (1  $\mu$ g) and T7 RNA polymerase (150 units) and/or T7 autogenes (0.3  $\mu$ g) pT7AUTO2C- or pCMV/T7-T7 pol complexed with 10 nmol of DC-chol:DOPE liposomes (3:2, mol/mol). In Figure 12A, transfection efficiency was measured as CAT activity determined 48 hours after transfection and in Figure 12B, toxicity was measured as protein recovered in transfected cells 48 hours after transfection as a percentage of protein recovered in untreated control cells.

Figures 13A and 13B show a comparison of two T7 autogenes, pT7 AUTO 2C and pCMV/T7-T7pol, for their ability to sustain pT7-CAT expression over time (Figure 13A) and their relative toxicities over time (Figure 13B). In both Figures 10A and 10B, 293 cells were transfected for 4 hours with 1  $\mu$ g pT7-CAT and pCMV/T7-T7pol (0.3  $\mu$ g)

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or with 1  $\mu$ g pT7-CAT, pT7 AUTO 2C (0.3  $\mu$ g) and 150 units of T7 RNA polymerase, complexed with 10 nmol of DC-Chol:DOPE (3:2, mol/mol) liposomes.

Figures 14A and 14B show a comparison of two T7 autogenes, pT7 AUTO 2C and pCMV/T7-T7pol, for their ability to sustain expression of pT7-CAT or fT7-CAT over time (Figure 14A) and their relative toxicities over time (Figure 14B). In both Figures 13A and 13B, 293 cells were transfected for 4 hours with 0.27 pmol pT7-CAT or fT7-CAT and pCMV/T7-T7pol (0.3  $\mu$ g) or with 0.27 pmol pT7-CAT or fT7-CAT, pT7 AUTO 2C (0.3  $\mu$ g) and 150 units of T7 RNA polymerase, complexed with 10 nmol of DC-Chol:DOPE (3:2, mol/mol) liposomes.

Figures 15A, 15B, 16A and 16B show CAT activity (Figures 15A and 15B) and protein recovered (Figures 16A and 16B) in 2008, C3, CHO and 293 cells transfected with pT7-CAT (1  $\mu$ g/well) co-delivered to cells with increasing concentrations of pT7 AUTO 2C- (Figures 15A and 16A) or pCMV/T7-T7pol (Figures 15B and 16B) by DC-chol:DOPE liposomes at 10 nmol lipid/ $\mu$ g DNA.

Figures 17A, 17B, 18A and 18B show CAT activity (Figures 17A and 17B) and protein recovered (Figures 18A and 18B) in 2008, C3, CHO and 293 cells transfected with pT7-CAT (1  $\mu$ g/well) co-delivered to cells with increasing concentrations of pT7 AUTO 2C (Figures 17A and 18A) or pCMV/T7-T7pol (Figures 17B and 18B) by LipofectAMINE at 5 nmol lipid/ $\mu$ g DNA.

Figure 19 shows a comparison of the CAT activities of plasmid (pT7-CAT and pCMV-CAT) and linear (fT7-CAT and fCMV-CAT) DNA sequences delivered by DC-chol:DOPE (3:2 mol/mol) liposomes or liposome/polylysine/DNA ternary complex (LPD). 0.27 pmol of DNA (pT7-CAT,

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PCM-V-CAT, fT7-CAT or fCMV-CAT) complexed with liposomal lipid (10 nmol lipid/ $\mu$ g DNA) or formulated in LPD complex, was used to transfect 293-T7 cells (a 293 cell line stably transfected with pCMV/T7-T7pol) for 4 hours. CAT assays were performed 48 hours following the transfection.

Figures 20A and 20B show CAT activity (Fig. 20A) in 293, BL6 and C3 cell lines. For the cytoplasmic expression system, 0.27 pmol/well of linear (fT7-CAT) or plasmid (pT7-CAT) DNA was codelivered to cells with pCMV/T7-T7 pol autogene (0.3  $\mu$ g/wells) complexed with DC-chol liposomes (Fig. 20A) or lipofectAMINE (Fig. 20B) (10 nmol lipid/ $\mu$ g DNA). For the nuclear expression system, either pCMV-CAT-CAT or fCMV-CAT (0.27 pmol/well) were complexed with either DC-Chol:DOPE liposomes (Fig. 20A) or lipofectAMINE (Fig. 20B) (10 nmol/lipid/ $\mu$ g) for delivery to cells. Cells were transfected for four hours and CAT activity was measured 48 hours after transfection.

Figures 21A and 21B: pCMV/T7-T7pol produces higher levels of CAT activity than pT7 AUTO 2C-autogene due to a higher production of T7 RNA polymerase. (21A) 1 $\mu$ g of pT7-CAT was co-transfected with either 1) 150 U of T7 RNA polymerase alone; 2) increasing concentrations of pCMV/T7-T7pol or pT7 AUTO 2C alone; or 3) increasing concentrations of pCMV/T7-T7Pol or pT7 AUTO 2C combined with 150 U of T7 RNA polymerase. CAT assays were performed on cell lysates 2 days after transfection. (21B) 0.3 $\mu$ g of pCMV/T7-T7pol or pT7 AUTO 2C was transfected alone or together with 150 U of T7 RNA polymerase enzyme to 293 cells via DC-chol:DOPE liposomes (1 $\mu$ g DNA/10nmol lipid). Cells were lysed 2 days after transfection. Western blot analysis of the cell lysates was performed by hybridization of the membrane with goat polyclonal anti-T7 RNA polymerase antibody followed by hybridization with rabbit anti-goat IgG antibody. Lane a:

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Positive control = 1U pure T7 RNA polymerase enzyme; lane b: Negative control = untreated cell lysate; lane c: pCMV/T7-T7pol; lane d: pCMV/T7-T7pol + T7 RNA polymerase; lane e: T7 AUTO 2C-; lane f: T7 AUTO 2C- + T7 RNA polymerase.

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Figures 22A and 22B: pCMV/T7-T7pol promotes high, sustained levels of T7 RNA polymerase and CAT expression. (22A) Time course analysis of CAT activity based on the same data shown in Figure 13A. The data of Figure 22A is presented as total CAT activity rather than per mg protein as shown in Figure 13A. 1 $\mu$ g of pT7-CAT was combined with either 0.3 $\mu$ g of pCMV/T7-T7pol or 0.3 $\mu$ g of pT7 AUTO 2C + 150 U of T7 RNA polymerase and transfected to 293 cells via DC-chol:DOPE liposomes (1 $\mu$ g DNA/10nmol lipid). Cells were lysed daily up to 7 days after transfection and split once (1:1) at day 4 when they were 100% confluent. CAT activity was determined on all cell lysates after day 7. (22B) Cell lysates from transfections in (22A) were subjected to Western blot analysis by hybridization with polyclonal goat anti-T7 RNA polymerase antibody followed by rabbit anti-goat IgG antibody. The volume of each band was determined by densitometry. These values were multiplied by the total protein recovered from each transfection/1000 to yield total T7 RNA polymerase in relative units. Total T7 RNA polymerase was undetectable for transfections with pT7 AUTO 2C. Lanes 1-4 : pCMV/T7-T7pol lysates 1, 3, 5 & 7 days after transfection; lanes 5-8: pT7 AUTO 2C + T7 RNA polymerase lysates 1, 3, 5 & 7 days after transfection, lane 9: Negative control = untreated cell lysate.

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Figure 23: pT7-CAT/pCMV/T7-T7pol (cytoplasmic expression) induces higher levels of CAT expression when compared with pCMV-CAT (nuclear expression). 1 $\mu$ g of pT7-CAT was combined with 0.3, 0.7, 1.0 or 2.0 $\mu$ g of pCMV/T7-

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T7pol and transfected to 293 cells by DC-chol:DOPE liposomes (1 $\mu$ g DNA/10nmol lipid) for 4 h at 37°C. For comparison, 1 $\mu$ g pCMV-CAT complexed with DC-chol:DOPE liposomes was delivered to 293 cells under the same conditions. CAT activities were determined 2 days after transfection.

Figure 24: Time Course Comparing pT7-CAT/pCMV/T7-T7pol (cytoplasmic expression) and pCMV-CAT (nuclear expression) in 293 cells. 1 $\mu$ g of pT7-CAT was transfected to 293 cells with either 0.7, 1.0 or 2.0 $\mu$ g of pCMV/T7-T7pol via DC-chol:DOPE liposomes (1 $\mu$ g DNA/10nmol lipid) for 4 h at 37°C. 1 $\mu$ g of pCMV-CAT was complexed to DC-chol:DOPE liposomes and delivered to 293 cells in the same manner. Cells were lysed daily up to 7 days after transfection and were split 1:1 at day 4 after transfection.

Figures 25A and 25B: DNA/Protamine/Liposome Complexes Can Enhance Can Enhance Expression in the Cytoplasmic Expression System. 1 $\mu$ g of pT7-CAT (25A) or pCMV-CAT (25B) was combined with 0 or 2  $\mu$ g of protamine before complexing with one of the following liposome formulations (1 $\mu$ g DNA/10nmol lipid): DOTAP (2 $\mu$ mol/ml), DOTAP:DOPE (2 $\mu$ mol/ml), LipofectAMINE (2.34 $\mu$ mol/ml), DC-chol:DOPE (2 $\mu$ mol/ml), and Lipofectin (1.6 $\mu$ mol/ml). These DNA/Protamine/Liposome complexes were transfected to 293 cells which stably express the pCMV/T7-T7pol autogene. CAT assay were performed 2 days after transfection.

Figure 26: Sequencing results for HindIII junction site in pCMV/T7-T7pol. The T7 gene 1 from pAR3126 was cloned as a HindIII/BamHI fragment into a pcDNA vector backbone. The sequence between the J brackets is the junction site. The sequence between the A brackets is the T7 promoter from pcDNA3 and sequence

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between the B brackets is the HindIII site.

Figure 27: Sequencing results for BamHI junction site in pCMV/T7-T7pol. The sequence between the J brackets is the junction site of the T7 gene 1 from PAR3126 cloned as a HindIII/BamHI fragment into the pcDNA3 vector. The sequence between the C brackets is the BamHI site.

Figure 28: HindIII/BamHI fragment sequence from PAR3126 containing the T7 gene 1.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a cytoplasmic gene expression system for use in eukaryotic cells. Gene transfer with cytoplasmic expression systems can be more advantageous than with nuclear expression systems for a number of reasons. First of all, many non-viral gene transfer vectors have been developed to improve DNA delivery to the cytoplasm of cells; however, there have been no significant advances in improving the nuclear transport of DNA. It has been previously shown that nuclear transport of cytoplasmic DNA is of very low efficiency (Capecchi (1980) Cell 22, 479-488). Therefore, the overall expression of DNA is hindered by inefficient transport of DNA into the nucleus where the transcription machinery resides.

Since the majority of the DNA delivered to cells remains in the cytoplasm, it would be beneficial if these genes could be expressed in the cytoplasm. Delivery of enzymes such as bacteriophage T7 RNA polymerase would drive the expression of genes which contained a cognate promoter such as the T7 promoter. This expression would be largely in the cytoplasm since the bacteriophage T7 RNA



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polymerases do not contain nuclear localization signals (Dunn et al. (1988) Gene 68, 259-266) and stay in the cytoplasm.

The cytoplasmic expression systems of the invention rely on a dual promoter prokaryotic expression in the cytoplasm of cells without the addition of exogenous prokaryotic RNA polymerase. This autogene contains a eukaryotic promoter which drives the initial expression of prokaryotic RNA polymerase in the nucleus. Though nuclear delivery of the autogene is thought to be poor, enough prokaryotic RNA polymerase can be produced in the cytoplasm via a second prokaryotic promoter within the autogene. Therefore, the expression of prokaryotic RNA polymerase from this autogene is mostly driven in the cytoplasm by the prokaryotic promoter. The continuous RNA polymerase generated from the autogene is then able to induce sustained expression of a co-transfected nucleic acid sequence operably linked to the same prokaryotic promoter. Such autogenes are therefore useful in, for example, gene therapy/transfer applications in which high and sustained cytoplasmic expression is desirable.

By "target sequence" as used throughout the specification and claims is meant a double-stranded DNA sequence whose expression is desired. Examples of suitable target sequences for use in the invention are sequences encoding antisense RNA molecules or sequences which encode proteins or peptides. Accordingly, where the target sequence encodes an antisense RNA molecule or ribozymemolecule, "expression" refers to transcription of the target sequence and where the target sequence encodes a protein or peptide, "expression" refers to transcription and translation of the target sequence. It is understood that the target sequence to be utilized in the cytoplasmic expression system of the invention may be linear or plasmid DNA.

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The autogene of the invention comprises, at a minimum, the following elements operatively linked: a nucleic acid sequence encoding a prokaryotic RNA polymerase gene, a cognate promoter of the RNA polymerase and a promoter capable of functioning in eukaryotic cells, e.g., a eukaryotic promoter. By "operatively linked" is meant that the elements are in a spatial arrangement which results in expression of the RNA polymerase at levels sufficient to initiate transcription, through the cognate promoter, of the nucleic acid sequence encoding the RNA polymerase.

It is to be understood that the autogenes of the invention may be in circular form as plasmid DNA or in linear form such as chemically synthesized DNA or PCR amplification products. In addition, the autogenes of the invention may be used to sustain the expression of the target sequence present in plasmid DNA.

The RNA polymerase encoded by the invention is preferably a single polypeptide enzyme that is capable of recognizing its cognate promoter sequence with high affinity and specificity and that does not require host cell factors to initiate transcription from the cognate promoter. Examples of RNA polymerases suitable for use in the autogenes of the invention include, but are not limited to, the bacteriophage RNA polymerases T7, SP6 and T3 and mitochondrial RNA polymerases.

The "eukaryotic promoter" of the autogenes of the invention may be any promoter which is capable of initiating transcription in the nucleus of eukaryotic cells.

By "cognate promoter" is meant any promoter sequence which is recognized by the RNA polymerase encoded by the autogene. The cognate promoter sequence may be a promoter sequence cloned from the genome of the RNA polymerase or it may be modified by mutations in order to reduce the level of expression of RNA polymerase from the

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autogene and hence, reduce toxicity of the autogenes to E. coli and mammalian cells.

In one embodiment, the autogene of the invention comprises in 5' to 3' order: the following elements operatively linked: a eukaryotic promoter, a cognate promoter for the RNA polymerase, and a nucleic acid sequence encoding the RNA polymerase. In a preferred embodiment, the RNA polymerase is bacteriophage T7 RNA polymerase, the cognate promoter is a T7 promoter and the eukaryotic promoter is a cytomegalovirus (CMV) promoter. An example of a preferred RNA polymerase autogene is the pCMV/T7-T7 pol autogene shown in Figure 2. This autogene has been demonstrated to be nontoxic to both E. coli and mammalian cells and supports the cytoplasmic expression of target sequences contained in both linear and plasmid DNA.

It is to be understood by those of ordinary skill in the art however, that levels of expression of RNA polymerase from the autogenes of the invention may be modulated by modification of the minimal elements present in the autogene (i.e. the eukaryotic promoter, the cognate promoter and the RNA polymerase gene) or by the insertion of additional elements into the autogene. For example, mutant RNA polymerases can be designed which are capable of binding to their cognate promoter and carrying on transcription without being lethal to a bacterial host (U.S. Patent 5,122,457) and the wild-type cognate promoters may be mutated (preferably by point mutations) to reduce transcription of the gene operably linked to the cognate promoter. Examples of mutations of the T7 promoter include, but are not limited to, m2T7, and M3T7p which have been shown to have 1% and 10% the activity of wild-type T7 promoter in in vitro transcription assays. Alternatively, the cognate promoter may be modified by attaching at its 3' end a bacterial operator sequence such as the lac operator where the operator may serve to reduce the toxicity of autogene constructs in the bacterial host.

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It is therefore contemplated that an autogene of the invention may comprise the following elements operatively linked: a promoter active in eukaryotic cells (e.g., a eukaryotic promoter) a modified cognate promoter and a nucleic acid sequence encoding the RNA polymerase.

5 In an alternative embodiment, the modified or unmodified cognate promoter may be linked at its 3' end to the 5' end of an internal ribosome entry site sequence (IRES) in order to enhance translation of the RNA polymerase gene by enabling the formation of translational  
10 initiation complex in eukaryotic cells without the need for the RNA to be capped at its 5' end. In a preferred embodiment, the IRES is a priornaviral IRES, most preferably an encephalomyocarditis (EMC) IRES sequence.

In yet another embodiment, the RNA polymerase  
15 gene may be linked at its 3' end to its cognate transcription terminator sequence in order to enhance the fidelity of transcription of the RNA polymerase gene. Such terminator sequences can be cloned from the genome encoding the RNA polymerase by, for example, physically  
20 mapping the 3' end of RNA transcripts by methods known to those of ordinary skill in the art or, where the sequence of the cognate terminator is known, the sequence may be synthesized. Examples of autogene constructs in which the cognate promoter is linked to an IRES and the 3' end of  
25 the polymerase gene is linked to a terminator sequence are shown in Figure 3 where the autogene construct comprises in 5' to 3' order: a CMV immediate early promoter, a mutant T7 promoter, an EMC IRES, sequence the T7 RNA polymerase gene and a T7 terminator and in Figure 4, where  
30 the autogene comprises in 5' to 3' order: a CMV promoter, a T7 promoter fused to the lac operator, an EMC IRES sequence, a T7 RNA polymerase gene, and a T7 terminator. Of course, those of ordinary skill in the art would recognize that since IRES sequences are translated poorly  
35 in bacteria, toxicity of autogenes containing an IRES is

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reduced and one could therefore substitute an unmodified wild-type T7 promoter for the lac-modified T7 promoter in the autogene construct shown in Figure 4. In addition, it is contemplated that an autogene construct of the invention may be designed to contain either an IRES or a terminator sequence or both.

The present invention also relates to cytoplasmic expression systems which utilize the RNA polymerase autogenes of the invention.

In one embodiment, the cytoplasmic expression system is a dual sequence cytoplasmic expression system which consists of a first nucleic acid sequence comprising a eukaryotic promoter operatively linked to a cognate promoter which is operatively linked to a prokaryotic RNA polymerase gene and a second sequence which comprises a second copy of the cognate promoter of the first sequence operatively linked to the target sequence.

In another embodiment, the present invention also provides a "single sequence cytoplasmic expression system" in which the first and second sequences described above are contained in a single nucleic acid molecule. An example of such single sequence expression system is provided in Figures 5 and 6.

As shown in Figure 5, it is also contemplated that in a single sequence system the first copy of the cognate promoter may be a mutant promoter and the second copy of the cognate promoter (driving expression of the target sequence) may be a wild-type promoter.

It is contemplated that the single sequence system may be more efficient for introduction into host cells than the dual system composed of two separate plasmids. However, the dual system may be of particular utility in situations where it is desirable to adjust the ratio of expressed RNA polymerase and the target sequence of interest by providing different ratios of the two plasmids. The present invention also relates to the use

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of the self-initiating and self-sustaining autogene in the above single sequence or double sequence expression systems to express other gene products including proteins, anti-sense molecules or ribozymes in eukaryotic cells.

Such expression systems encompass a wide variety of applications. For example, it is especially suitable for expressing genes in a specific cell type where appropriate promoters for that cell type are unavailable. The autogenes may therefore be used for synthesizing desired proteins in vitro or in vivo. It is also useful in vitro for the transient expression of a newly cloned gene for confirmation of the identity of its encoded gene product as well as the rapid production of high amounts of proteins for use in immunization of animals for the generation of specific antibodies. In this regard, the present invention may be used to introduce exogenous genes into cells and tissues in vivo for transient expression of the gene product to elicit an antigen-specific host immune response. The short-term nature of gene expression may be ideal for its use as a vehicle for in vivo immunization of a host to an antigen.

A major impediment in the current attempts of gene therapy is the integration of foreign genes in non-dividing eukaryotic host cells. The ability of the present system to permit gene expression in the cell cytoplasm circumvents this potential problem, as gene expression can be achieved in quiescent cells. However, as the DNA construct is gradually degraded in the cytoplasm or diluted in number when cells divide over time, the present invention is also self-limiting. The self-limiting nature of this expression system is particularly suitable for use in settings where gene expression is desirable only temporarily or transiently. For example, the present invention may be used to target lymphokines genes to tumor sites in vivo for the in situ activation of cytotoxic lymphocytes to mediate tumor

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cytolysis. Since the expression of the exogenous genes is transient, the uptake of DNA by cells not lysed in the process would not eventually induce permanent and sustained synthesis of lymphokines leading to potentially deleterious consequences, such as autoimmunity.

5           The single or dual sequence expression systems may be suspended in saline, PBS, serum free media or any aqueous solution suitable for in vivo administration. The solution may be injected into an animal, including a human, intravenously, intramuscularly, intracranially, 10 subcutaneously or directly into a tissue or organ. The single or dual sequence systems may be injected alone, encapsulated by liposomes or linked to any carrier molecules capable of translocating the system across the plasma membrane. For in vivo administration, the 15 expression system may be injected in the dose range of 0.01-100 mg/kg. If the expression product of the target sequence is desired to be secreted into the bloodstream, the systems may be injected intravenously to cause endothelial cell uptake and release of the expression 20 product directly into the circulation.

Any articles or patents referenced herein are hereby incorporated by reference. The following example illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

25

#### EXAMPLES

##### Abbreviations

30   PCR: polymerase chain reaction; CAT: E coli chloramphenicol acetyltransferase; CMV: cytomegalovirus immediate early promoter; T7: bacteriophage T7 promoter; T7pol: bacteriophage T7 RNA polymerase coding gene; DC-chol: 3 $\beta$ [N-(N',N'-dimethylaminoethane)-carbamoyl] 35 cholesterol; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphatidyl-

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ethanolamine; LPD: liposome/polycation/DNA ternary complex; CHEMS: cholesteryl hemisuccinate

### Materials.

T7 RNA polymerase (New England Biolabs, Beverly, MA) was used without further purification. All the chemicals for PCR including Taq polymerase, nucleotides and buffer were purchased from Gibco BRL (Gaithersburg, MD). Acetyl coenzyme A, chloramphenicol, Triton X-100 and the hydrobromide salt of the polycation poly-L-lysine (MW 25,600) were from Sigma (St Louis, MO). [<sup>3</sup>H]acetyl coenzyme A and Beta-Max scintillation cocktail were from ICN Biomedicals (Costa Mesa, CA).

Dioleoylphosphatidylethanolamine (DOPE) and cholesteryl-hemisuccinate (CHEMS) were obtained from Avanti Polar Lipids, Inc (Birmingham, AL). 3β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol) was synthesized according to Gao and Huang ((1991) Biochem. Biophys. Res. Commun. 179: 280-285). Unilamellar DC-chol:DOPE liposomes of approximately 150 nm in diameter were prepared by microfluidization of a hydrated mixture of DC-chol and DOPE (3:2, m/m), and filter sterilized. pH-sensitive liposomes (approximately 120 nm in diameter) composed of CHEMS and DOPE (3:2, mol/mol) were prepared by sonication. LipofectAMINE was obtained from Gibco BRL.

### PCR amplified linear DNA fragments and Plasmid DNA

For PCR, 10 ng of plasmid template, 50 pmoles of each primer (shown in Figure 1), 0.2 mM dNTPs, and 2.5 units of Thermus aquaticus (Taq) DNA polymerase were used. The PCR protocol consists of 4 min of denaturation at 94°C, then 30 cycles of 45 sec at 94°C, 1 min at 68°C, and 2 min at 72°C and finally extension of 5 min at 72°C. The resulting PCR amplified fragments were extracted with phenol-chloroform, ethanol-precipitated, resuspended in water and then further purified by filtration through a



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size exclusion filter to remove unused primers and free oligonucleotide.

Plasmid pT7-CAT which is the same as pT7-EMC-CAT, a plasmid containing the CAT reporter gene driven by the bacteriophage T7 promoter (Elroy-Stein, et al. (1989) Proc. Natl. Acad. Sci. USA 86: 6126-6130), was a kind gift provided by Dr. B. Moss (National Institutes of Health). pT7 AUTO 2C, an autogene composed of the T7 RNA polymerase gene and its corresponding promoter, was maintained and purified as previously described by Gao et al (Gao, X., et al. (1994) Biochem. Biophys. Res. Commun. 200: 1201-1206). Plasmid pUCCMV-CAT was constructed by Dr. H. Farhood (Farhood, H., et al. (1995) Biochem. Biophys. Acta., 1235: 289-295). The pCMV/T7-T7pol autogene was constructed by inserting a HindIII/BamHI fragment from pAR3126 (Dunn, J.J., et al. (1988) Gene 68: 259-266) containing the cDNA of T7 RNA polymerase into the corresponding sites of the pCDNA3 plasmid vector (Invitrogen Corp., San Diego, CA) (See Figures 2, 26-28). This autogene was amplified in E. coli DH5 $\alpha$ F' cells and purified by CsCl gradient centrifugation method (Sambrook, J., et al. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: Plainview, NY).

#### Preparation of LPD complex

The molecular weight of the hydrobromide salt of poly-L-lysine used to produce the LPD was 25,600. LPD complex was prepared prior to transfection by mixing pH-sensitive liposomes containing CHEMS and DOPE and the cationic DNA/polylysine (1:0.75, w/w) complex. The lipid to DNA ratio in the final LPD complex was 3:1 (w/w). The resulting LPD complex had a cationic charge.

#### Tissue culture

Human embryonic kidney 293 cells, BL6 murine melanoma cells, 2008 ovarian carcinoma cells, CHO Chinese

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Hamster Ovary Cells, and C3 HeLa cells, all of which were routinely tested and found to be free of mycoplasma, were cultured in DMEM medium for 293 cells, RPMI medium for BL6, C3 and 2008 cells and F12 medium for CHO cells. All of the medium was supplemented with 10% fetal bovine serum, penicillin (200 units/ml) and streptomycin (100 ug/ml) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. 293-T7, a 293 cell line which produces endogenous T7 RNA polymerase, was established by co-transfecting 293 cells with pCMV/T7-T7pol and pBK-CMV (Neo<sup>r</sup>) phagemid followed by selection by G418. The 293-T7 cells were then cultured in DMEM medium (with serum and antibiotics as for 293 cells) supplemented with G418 (0.4 mg/ml).

Transfection Protocol For Figures 7-11 and 19

Cells (293 or 293-T7 cells) cultured in 24 well plates (about 70-80% confluent) were used for transfection. DNA (plasmid or PCR fragment), complexed with DC-chol:DOPE liposomes (10 nmol lipid/ $\mu$ g DNA) or formulated in LPD complex, was used to transfect the cells. For 293 cells, pCMV/T7-T7pol (as a source of T7 RNA polymerase) was co-delivered with T7-EMC-CAT (plasmid or PCR fragment). Four hours following the transfection, the medium (serum-free media) was replaced with normal growth medium (containing 10% fetal bovine serum and antibiotics), and cells were cultured for 2 days before the CAT assay was performed. For the time course study shown in Figure 5, cells were collected daily for 9 days, and were split 1:1 once on day 4 when they were 100% confluent.

Transfection Protocol for Figures 12-14

293 cells were grown to 70-80% confluency in 24 well plates. pT7-CAT (1 $\mu$ g) diluted in serum free DMEM medium was mixed with either T7 RNA polymerase (150 U, New England Biolabs, Beverly, MA), 0.3  $\mu$ g of either autogene

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(pCMV/T7-T7pol or pT7 AUTO 2C) or a combination of enzyme and autogene before complexing with DC-chol:DOPE liposomes (10 nmol total lipid). This mixture was used to transfect cells for 4 hours at 37°C after which transfection medium (serum-free medium) was replaced with growth medium (DMEM containing 10% FBS plus antibiotics). For transfection efficiency studies, cells were lysed for CAT assays 48 hours following transfection. For time course studies, cells were split once at day 4 and were collected daily up to 7 days after transfection for CAT assay.

#### Transfection for Figures 15-18

2008, C3, 293, and CHO cell lines were cultured to ~70% confluency in 24 well plates prior to transfection.

pT7-CAT (1µg/well) was co-delivered to cells with increasing concentrations of pCMV/T7-T7pol or pT7 AUTO2C- autogene T7 RNA polymerase (150U) by either DC-chol liposomes (10nmol lipid/mgDNA) or LipofectAmine (5 nmol lipid/µg DNA). After 4 hr, transfection medium was replaced with growth medium and cells were lysed 48 hr later.

#### Transfection Protocol For Figures 20A and 20B

293, BL6, and C3 cells were cultured in 24 well plates until ~80% confluency prior to transfection. For the cytoplasmic expression system, either pT7-CAT or fT7-CAT (0.27 pmol/well) was co-delivered to cells with pCMV/T7-T7pol autogene (0.3 µg/well) via DC-chol liposomes or LipofectAMINE (10 nmol lipid/µg DNA). For the nuclear expression system, either pCMV-CAT or fCMV-CAT (0.27 pmol/well) were complexed with either DC-chol liposomes or LipofectAMINE (10 nmol lipid/µg DNA) for cell delivery. Cells were transfected for 4 hr before replacement of transfection medium with culture medium. Cells were then lysed 48 hr after transfection.

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° Transfection Protocol For Figures 21-25

Transfection of cells was performed as described in the Brief Description of Drawings for Figures 21 through 25 using a standard incubation protocol of 4 hours 37°C as described for Figures 12-14.

5 CAT Assay For All Examples

Following transfection, cells were washed once with PBS and lysed with 200 µl of lysis buffer consisting of 0.1% Triton X-100 (Sigma, St. Louis, MO) in 200 mM Tris-HCl (pH 7.8). CAT assays were performed according to the methods described by Sankaran (Sankaran, K. (1992) Anal. Biochem., 200: 180-186) with a few modifications. 1 mM chloramphenicol (Sigma, St. Louis, MO), 0.1 mM acetyl CoA (Sigma, St. Louis, MO), and 0.1 µCi [<sup>3</sup>H] acetyl CoA (ICN Biomedicals, Costa Mesa, CA) were used for the assay mixture. The reaction was performed at 37°C for 1 hour and the product of the reaction, acetylated chloramphenicol (1,3-diacetylchloramphenicol), was then extracted from the aqueous phase with 1 ml of toluene. One half of the organic phase was then mixed with 3 ml of Beta-Max™ (ICN Biomedicals, Costa Mesa, CA) and counted for radioactivity. One unit was defined as the amount of enzyme converting 1 nmol of acetyl groups to chloramphenicol per minute under the above reaction conditions.

Example 1

Cytoplasmic Expression of the CAT reporter gene in 293-T7 cells as a function of pT7-CAT or fT7-CAT DNA concentration

30 Cytoplasmic expression of T7-CAT DNAs was investigated by examining expression of the CAT reporter gene in 293-T7 cells as a function of pT7-CAT or fT7-CAT DNA concentrations.

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As shown in Figure 7, the CAT activity increased with increasing amounts of either pT7-CAT or fT7-CAT, but reached a plateau when the T7-CAT DNA concentrations were above 0.54 pmol. No significant difference was found between CAT activity for pT7-CAT and fT7-CAT when delivered by DC-chol:DOPE liposomes.

The failure to observe a further increase of CAT activity with continuous increase in the amount of T7-CAT DNA might be due to the amount of T7 RNA polymerase produced in 293-T7 cells since probably only those 293-T7 cells which produce moderate amounts of enzyme could survive the selection after co-transfection.

#### Example 2

##### Cytoplasmic Expression of CAT reporter gene in 293-T7 cells as a function of time

As shown in Figure 8, CAT expression from pT7-CAT or fT7-CAT peaked at day 5 and declined slowly thereafter to a level of expression at day 9 that was about 1/4 of the peak level. This duration of CAT expression is longer than that seen with the nuclear expression system which is usually 3-5 days (Gao, X., et al. (1994) Biochem. Biophys. Res. Commun., 200: 1201-1206); a result that might be due to the fact that some of the T7-CAT remained associated with liposomes following release into the cytoplasm thereby preventing the DNA from being rapidly degraded by cytoplasmic enzymes.

#### Example 3

##### Cytoplasmic Expression of CAT Reported Gene In Normal 293 Cells

In order to determine if the CAT Reporter Gene could be expressed in 293 cells which do not stably express T7 RNA polymerase, pT7-CAT or fT7-CAT were co-

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delivered into 293 cells with either purified T7 RNA polymerase or the T7 autogene pCMV/T7-T7pol. The data presented in Table 1 clearly show that CAT expression for both plasmid and linear DNA fragments were dramatically lower when the source of T7 RNA polymerase was the exogenously supplied pure protein as compared to the T7 autogene.

Table 1

| Source of T7 RNA polymerase | CAT activity (U/mg) |             |
|-----------------------------|---------------------|-------------|
|                             | pT7-CAT             | fT7-CAT     |
| Pure Protein                | 0.18 ± 0.02         | 0.17 ± 0.01 |
| pCMV/T7-T7pol               | 6.42 ± 0.37         | 5.53 ± 0.73 |

<sup>a</sup>T7-CAT (1 µg) was co-transfected with either pure T7 RNA polymerase (150U/well) or pCMV/T7-T7pol (0.3 µg/well) to 293 cells with DC-chol:DOPE liposomes (10 nmol) for 4 hours. CAT activity was assayed 48 h after transfection.

Thus, the pCMV/T7-T7pol autogene was employed as a source of T7 RNA polymerase in subsequent studies.

#### Example 4

#### CAT Activity As A Function Of pCMV/T7-T7pol Autogene Concentration

As shown in Figure 9, the expression of CAT activity from pT7-CAT and fT7-CAT increased steadily with increasing amounts of pCMV/T7-T7pol autogene. Moreover, the transfection efficiency of fT7-CAT was comparable to that of pT7-CAT at all concentrations of autogene tested and no toxicity was noticed at any ratios examined if the cell confluence was above 70% at the time of transfection. At low cell confluency, toxicity was noticed if the molar ratio was above 3/1. Thus, a molar ratio of autogene to T7-CAT of 5/1 was chosen for the subsequent studies.

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Example 5Cytoplasmic Expression Of CAT Reporter Gene  
As A Function Of T7-CAT DNA Concentration

As Figure 10 shows, CAT activity increased in  
5 normal 293 cells co-transfected with varying amounts of  
pT7-CAT or fT7-CAT and pCMV/T7-T7pol (0.3mg) in a fashion  
slightly different from that observed earlier in 293-T7  
cells. This result might be due to the difference in the  
amount of intracellular T7 RNA polymerase expressed in 293  
10 and T7-293 cells. No toxicity was found at any dose  
tested.

Example 6Cytoplasmic Expression of CAT Reporter Gene In 293  
15 Cells As A Function of Time

As shown in Figure 11A, high and sustained CAT  
expression was found with both pT7-CAT and fT7-CAT over  
time. Indeed, the level of expression with fT7-CAT was  
20 slightly greater than that with pT7-CAT. In addition, the  
protein recovery over time of fT7-CAT and pT7-CAT were  
comparable (Fig. 11B) indicating no toxicity.

Example 7Comparison Of Cytoplasmic Expression  
25 Of pT7-CAT And fT7-CAT Using Either The  
pCMV/T7-T7pol Autogene Or The pT7AUTO2C Autogene

As shown in Figure 12A, pCMV/T7-T7pol produced a  
30 higher level of CAT gene expression as compared with that  
seen with the pT7AUTO2C autogene. This elevated CAT gene  
expression with the pCMV/T7-T7pol autogene did not require  
any additional T7 RNA polymerase to drive the T7 RNA  
polymerase regeneration process. However, when additional  
35 enzyme was added, there was a further increase in CAT

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activity, suggesting that exogenous T7 RNA polymerase delivered to the cytoplasm is acting on the T7 promoter to promote autogene expression and thus induce higher reporter gene expression than that seen with pCMV/T7-T7pol alone. These results are in sharp contrast with that seen with pT7AUTO2C-, which is completely dependent on the presence of exogenously added T7 RNA polymerase enzyme to initiate the autocatalytic production of T7 RNA.

CAT activity with pT7AUTO2C was only seen in the presence of additional enzyme and the CAT activity was lower than that observed with the pCMV/T7-T7pol autogene. Both autogenes, however, demonstrated much higher CAT activity than that seen with T7 RNA polymerase alone. This result suggests that a constant production of T7 RNA polymerase is required for higher levels of gene expression in the cytoplasm.

As shown in Figure 12B, protein recovery was nearly the same for all of the transfection formulations, thereby demonstrating that all of the formulations produced no toxicity in 293 cells.

#### Example 8

##### Time Course of Cytoplasmic Expression of the CAT Reporter Gene Using Either pT7 Auto 2C Or pCMV/T7-T7pol As The T7 RNA Polymerase Autogene

To test whether co-delivery of pT7-CAT with either pCMV/T7-T7pol or pT7AUTO2C would produce CAT expression over longer periods of time, a time course study was conducted comparing the effects pCMV/T7-T7pol and pT7AUTO2C on pT7-CAT or pT7-CAT expression over time. As shown in Figure 13A, pCMV/T7-T7pol produced continuously higher levels of pT7-CAT expression than that seen with pT7AUTO2C over a 7 day time period. When expressed on a mg protein basis, CAT expression with pCMV/T7-T7pol increased up to 5 days following



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transfection and declined thereafter with activity still remaining high at day 7. In contrast, CAT expression seen with pT7AUTO2C was lower than that seen with the pCMV/T7-T7pol autogene and only remained elevated up to day 3 at which time it declined to baseline levels by day 6. This result is consistent with time course data seen in previous experiments with pT7AUTO2C by Gao et al. ((1994) Biochem. Biophys. Res. Commun. 200: 1201-1206).

As Figure 14A shows, fT7-CAT, while showing a slightly lower level of CAT expression than pT7-CAT, exhibited the same pattern as of CAT expression over time as did pT7-CAT when co-delivered with pCMV/T7pol autogene. In contrast, no CAT expression was observed when fT7-CAT was delivered with T7 AUTO 2C autogene (Figure 14A). In addition, the protein recovery data in Figures 13B and 14B indicate that cells treated with pT7AUTO2C did not grow as well as the cells treated with pCMV/T7-T7pol, thereby suggesting that pT7AUTO2C was somewhat more toxic to cells than pCMV/T7-T7pol.

When expressed as total CAT activity without standardizing the values on a per mg protein basis, the CAT activity in pCMV/T7-T7 pol transfected cells did not show the decrease in activity after 5 days (Figure 22A). Standardizing values for protein may not accurately reflect CAT expression in transfected cells because non-transfected cells also present in the culture continue to proliferate, thus contributing to the total amount of protein present and diluting the total CAT activity in the transfected cells. Cells were split (1:1) when they were 100% confluent on day 4 (B) and total CAT activity is based on the average of three culture wells containing transfected cells.

Western blot analysis was used to investigate the sustained CAT activity in pCMV/T7-T7 pol compared to pT7 AUTO 2C transfected cells which exhibited a decrease after an initial transient increase in CAT activity.

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Cells transfected with pCMV/T7-T7pol produced the highest levels of T7 RNA polymerase between days 3 and 5 after transfection (Figure 22B). This is consistent with fact that CAT activity increases consistently through day 5 after transfection. CAT activity fails to increase after day 5 due to a decrease in T7 RNA polymerase transcription which limits CAT transcription. The levels of T7 RNA polymerase transcribed by T7 AUTO 2C were again too low to be detected.

#### Example 9

pCMV/T7-T7pol autogene is less toxic and more efficient in its expression of pT7-CAT than pT7AUTO2C in 4 different cell lines

The efficiency and toxicity of pCMV/T7-T7pol or T7 AUTO 2C autogenes co-delivered with pT7-CAT was compared in 2008, C3, CHO, and 293 cells in Figures 15-18. It is obvious that the pCMV/T7-T7pol autogene induced considerably higher levels of CAT expression in all the cell lines used as compared to pT7AUTO2C (compare Figures 15B and 17B with Figures 15A and 17A). This expression seems to be formulation dependent since LipofectAMINE seemed to induce even higher levels of CAT activity than that seen with DC-chol liposomes for both autogenes (compare Figures 14A and 14B with Figures 15A and 15B). Though 2008 cells demonstrated low levels of CAT expression with DC-chol liposomes and no expression with LipofectAMINE, it has been shown in our laboratory that optimization of the delivery formulation with pCMV/T7-T7pol autogene allows for moderate CAT expression in these cells (data not shown). These results confirm that pCMV/T7-T7pol autogene can induce a higher CAT expression in several different cell lines than that seen with pT7AUTO2C autogene. The reason for higher CAT activity with LipofectAMINE is unclear at this time; however, it

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could be due to the multivalent positive charge of LipofectAMINE and its ability to further condense DNA for cellular entry.

When comparing protein recovery in all cell lines (Figure 16A, 16B), it is apparent that pT7AUTO2C autogene delivered with DC-chol liposomes is toxic to all cells (50% loss of cells) except 293 cells (Figure 16A). In contrast, pCMV/T7-T7pol autogene delivered with DC-chol was only toxic to 2008 cells (Figure 16B). The high toxicity to 2008 cells in these transfections is not understood at this time.

When these autogenes were delivered to these cells by LipofectAMINE, toxicity was observed with C3 and CHO cells (50% loss of cells) (Figures 18A and 18B). pCMV/T7-T7pol autogene delivered with LipofectAMINE to C3 and CHO cells showed a dose dependent increase in toxicity probably due to increasing LipofectAMINE concentrations with increasing autogene concentrations (Figure 18B). No toxicity was observed with 2008 cells with LipofectAMINE probably because these cells were not transfectable with this formulation. None of the formulations tested were toxic to 293 cells. In all of the cell lines tested, the toxicity of the autogenes was dependent on the cell confluency prior to transfection since higher cell confluencies demonstrated less toxicity.

The reasons for the toxicity due to the pT7AUTO2C autogene are unclear. It is assumed that the toxicity could be due to either extremely high levels of T7 RNA polymerase generated in the transfected cells or because of exogenous T7 RNA polymerase not transfected to cells. Experiments are now in progress to determine the levels of T7 RNA polymerase produced in cells transfected with these autogenes.

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Example 10Comparison of the Transfectability of plasmid  
in Nuclear and Cytoplasmic Expression Systems

5 The transfectability of plasmid DNA (pT7-CAT or pCMV-CAT) and linear PCR-generated DNA fragments (fT7-CAT or fCMV-CAT) in cytoplasmic (T7-CAT) and nuclear expression systems (CMV-CAT) were compared in a cell line (293-T7) which produces endogenous T7 RNA polymerase.

10 In brief, plasmid or linear T7-CAT and CMV-CAT DNAs were delivered to 293-T7 cells by DC-chol:DOPE liposomes or LPD and CAT activities were then determined 48 hours after transfection. The results are shown in Figure 19.

15 The results demonstrate that strong CAT gene expression, comparable to that observed for pT7-CAT and for pCMV-CAT was found in cells transfected with fT7-CAT if delivered by DC-chol:DOPE liposomes while less CAT activity was detected if fT7-CAT was delivered by LPD.

20 The differential results between LPD and liposomes for fT7-CAT might be due to the fact that linear DNA may be easily condensed due to the lack of structural constraints seen with supercoiled DNA. Thus, highly condensed DNA, such as fT7-CAT condensed by polylysine in the LPD complex, may be too highly condensed to allow efficient un-coating of DNA in the cytoplasm for transcription. This point was indirectly confirmed by an in vitro transcription assay which showed that the transcription efficiency of pT7-CAT was decreased after its interaction with liposomes. Co-plexation of fT7-CAT with polylysine completely inhibited its accessibility to T7 RNA polymerase (data not shown).

30 With respect to transfectability of the nuclear expression system (pCMV-CAT and fCMV-CAT), very limited expression was obtained for fCMV-CAT delivered by either DC-chol DOPE liposomes or LPD when compared with pCMV CAT

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on a molar basis. This could be due to inefficient transport of linear DNA (fCMV-CAT) across the nuclear membrane.

#### Example 11

##### Comparison of the LipofectAMINE and DC-Chol Liposomes as Delivery Vehicles for transfection in both Nuclear and Cytoplasmic Expression Systems

To determine if the low CAT expression obtained in Example 9 for fCMV-CAT relative to that observed for pCMV-CAT might have resulted from the lack of an optimized delivery system for linear DNA transport to the nucleus, the following experiment was conducted: pCMV-CAT or fCMV-CAT (nuclear expression system) or pT7-CAT or fT7-CAT (cytoplasmic expression system) codelivered with pCMV/T7-T7pol autogene were delivered to 293, BL6 or C3 cells by either DC-chol liposomes or lipofectAMINE (10 nmol lipid/1 µg DNA), and CAT activity was assayed 48 hours after transfection. The results presented in Figures 20A (DC-chol liposomes) and 20B (lipofectAMINE) demonstrate that overall, lipofectAMINE prove to be a better delivery vehicle for transfection in both nuclear and cytoplasmic expression systems in the cell lines examined.

#### Example 12

##### Cytoplasmic Expression Using A Single Sequence Expression System Capable of Both Self Amplification of T7 RNA Polymerase And the Expression of Reporter Gene Driven by the T7 Promoter

293 cells are transfected with the single sequence expression system shown in Figure 12, either in circular plasmid form (as shown in the Figure), or in linear form, using DC-chol:DOPE liposomes. CAT activity is determined 48 hours after transfection.

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Example 13In Vivo Cotransfection With  
Transgenes Driven by the T7 Promoter  
and with pCMV/T7-T7pol Autogene Sequences

5 Mice are injected intravenously,  
intramuscularly, intracranially, subcutaneously or  
directly into the tissue or organ with complexes of DC-  
chol liposomes and transgenes driven by the T7 promoter  
(plasmid or linear) alone or combined with pCMV/T7-T7pol  
10 autogene (plasmid or linear). About 100 µg total DNA is  
injected. Animals are sacrificed 2 days later and  
extracts of tissues of interest (liver, kidney, lung,  
heart, brain, etc.) are assayed for CAT activity. CAT  
activity is determined in various tissues.

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Example 14pCMV/T7-T7pol Induces High Levels of Endogenous  
T7 RNA Polymerase and CAT Activity Without  
The Need For Exogenous T7 RNA Polymerase Enzyme

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When 293 cells are transfected with pT7-CAT +  
pCMV/T7-T7pol, high CAT activity was observed which was  
dependent on autogene concentration (Figure 21A).  
pCMV/T7-T7pol was not dependent on exogenous T7 RNA  
polymerase to initiate transcription since high CAT  
25 activity is observed without exogenous enzyme. pT7 AUTO  
2C, however, induced no CAT activity in transfected cells  
in the absence of exogenous T7 RNA polymerase enzyme. In  
addition, the activity observed with pT7 AUTO 2C was not  
as significant as that seen with pCMV/T7-T7pol.

30

Since CAT activity observed with pCMV-T7-T7pol  
was dose dependent and higher than that observed with pT7  
AUTO 2C, we investigated the possibility that pCMV/T7-  
T7pol may induce higher levels of T7 RNA polymerase to  
enhance expression of pT7-CAT. 293 cells transfected with  
35

- 35 -

° pCMV/T7-T7pol or pT7 AUTO 2C in the presence or absence of exogenous T7 RNA polymerase were lysed and subjected to Western blot analysis and probed with an anti-T7 RNA polymerase polyclonal antibody (Figure 21B). The results indicate that pCMV/T7-T7pol can produce high levels of endogenous T7 RNA polymerase in the presence or absence of exogenous T7 RNA polymerase; however, endogenous T7 RNA polymerase transcribed from pT7 AUTO 2C was too low to be detected. Therefore, the pCMV/T7-T7pol autogene can induce high CAT activity in cells transfected with pT7-CAT due to the high production of T7 RNA polymerase transcribed by pCMV/T7-T7pol.

#### Example 15

#### The pCMV-T7-T7 pol Cytoplasmic Expression System Can Produce Higher Levels of Activity Than Other Cytoplasmic or Nuclear Expression Systems

The cytoplasmic expression system pT7-CAT + pCMV/T7-T7pol was compared with a nuclear expression vector pCMV-CAT to determine if the cytoplasmic system can increase transcription/translation of the reporter gene CAT over that seen with the strong nuclear cytomegalovirus promoter. The addition of pCMV/T7-T7pol at concentrations above 0.3  $\mu$ g produced higher CAT activity than pCMV/CAT. Figure 23. CAT activity increased with increasing autogene concentration up to 2.0  $\mu$ g of pCMV/T7-T7pol. When considering these results and the comparisons with pT7 AUTO 2C, it is apparent that our cytoplasmic expression system with pCMV/T7-T7pol can produce higher levels of reporter gene expression than previously used cytoplasmic and nuclear expression systems.

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Example 16Transfection with pCMV/T7-T7 pol Consistently Produced  
Higher Levels of CAT Activity Compared To Transfection  
With pT7-CAT

5           When the cytoplasmic expression system pT7-CAT +  
pCMV/T7-T7pol was compared with pCMV-CAT in a time course  
study, the cytoplasmic system consistently produced higher  
CAT activities at all autogene concentrations used (0.7,  
1.0, 2.0 µg) over the 7 day period. Figure 24. The  
10 activity was strongest for the cytoplasmic system at days  
1 and 2 and then continued to fall through day 7. The  
highest level of activity seen with pCMV-CAT was seen  
around day 3-4 transfection. This is probably due to the  
fact that high concentrations of autogene produce rapid,  
15 high levels of T7 RNA polymerase to therefore induce high  
level of reporter gene expression in the cytoplasm.

Example 17Protamine Increases The Transfection  
Activity of Certain Lipid Delivery Formulations

20           Efficient transfection of both the cytoplasmic  
and nuclear expression systems depends on the type of  
liposome formulation used to transfect cells. When  
comparing delivery of pT7-CAT (cytoplasmic) (Figure 25A)  
25 or pCMV-CAT (nuclear) (Figure 25B), DOTAP and  
LipofectAMINE are very efficient in producing high levels  
of CAT activity in both systems. Other formulations such  
as DOTAP:DOPE, DC-chol:DOPE and Lipofectin are less  
effective in producing high CAT activity. However, the  
30 addition of protamine, a highly positively charged short  
peptide, to DNA before liposome addition enhanced the CAT  
activities seen with the less effective liposomes to  
levels comparable with DOTAP and LipofectAMINE. Protamine  
35 had no effect on transfections with DOTAP and



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- ° LipofectinAMINE. Figure 25A. Without being bound by theory, protamine may enhance transfection efficiency by condensing DNA before the addition of liposomes. Therefore, CAT activities may be enhanced with the cytoplasmic expression system of our invention by  
5 optimizing the formulation used to deliver the DNA to cells.

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WE CLAIM:

1. An autoregulatory nucleic acid sequence,  
said sequence comprising a eukaryotic promoter operatively  
linked to a prokaryotic promoter which is operatively  
5 linked to a nucleic acid sequence encoding a prokaryotic  
RNA polymerase which initiates transcription, through said  
prokaryotic promoter, of said nucleic acid sequence  
encoding said polymerase.
- 10 2. The autoregulatory sequence of claim 1,  
wherein said polymerase is a bacteriophage RNA polymerase.
3. The autoregulatory sequence of claim 1,  
wherein the second promoter is selected from the group  
15 consisting of T7, SP6 and T7 bacteriophage promoters.
4. The autoregulatory sequence of claim 2,  
wherein the eukaryotic promoter is a CMV promoter, the  
prokaryotic promoter is a T7 promoter and the polymerase  
20 is a bacteriophage T7 RNA polymerase.
5. The autoregulatory sequence of claim 1,  
wherein said sequence is linear.
- 25 6. The autoregulatory sequence of claim 1,  
wherein said sequence is contained in a plasmid expression  
vector.
7. The sequence of claim 5, wherein said  
30 eukaryotic promoter and said prokaryotic promoter are 5'  
of said nucleic acid sequence.
8. The sequence of claim 5, wherein said  
eukaryotic promoter is 5' of said prokaryotic promoter.

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9. An autoregulatory nucleic acid sequence comprising a target sequence operatively linked to the sequence of claim 1.

10. The autoregulatory sequence of claim 1, further comprising a second copy of the prokaryotic promoter operatively linked to a target sequence.

11. The sequence of claim 10, wherein an internal ribosome entry site is located between the second prokaryotic promoter the target sequence.

12. The sequence of any one of claims 9-11 wherein the target sequence encodes an antisense RNA molecule.

13. The sequence of any one of claims 9-11, wherein the target sequence encodes a protein.

14. The sequence of claim 13, wherein the protein is immunogenic.

15. The nucleic acid sequence of any one of claims 9-11, wherein said sequence is linear.

16. A method for expressing a target sequence in cells, said method comprising introducing into said cells with

an autoregulatory sequence comprising a eukaryotic promoter operatively linked to a prokaryotic promoter which is operatively linked to a nucleic acid sequence encoding a prokaryotic RNA polymerase which initiates transcription, through the prokaryotic promoter, of the sequence encoding the RNA polymerase; and

- 40 -

a second sequence comprising a second copy of the prokaryotic promoter operatively linked to the target sequence.

17. The method of claim 16, wherein the autoregulatory sequence and the second sequence are introduced into said cells as a lipid complex comprising a cationic lipid suitable for transfecting cells.

18. The method of claim 17, wherein the lipid complex further comprises a polycation.

19. The method of any one of claims 16-18 wherein the autoregulatory sequence and the second sequence are linear.

20. The method of claim 19, wherein the sequences are chemically synthesized.

21. A method for expressing a target sequence in cells, said method comprising introducing into said cells a single sequence comprising an autoregulatory sequence having a eukaryotic promoter operatively linked to a prokaryotic promoter which is operatively linked to a nucleic acid sequence encoding a product which initiates transcription, through said second promoter, of both the target sequence and the sequence encoding the RNA polymerase.

22. The method of claim 21, wherein the lipid complex further comprises a polycation.

23. The method of any one of claims 21-22, wherein the single sequence is linear.

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24. The method of claim 24, wherein the single sequence is chemically synthesized.

25. The method of claim 21, wherein the single sequence is introduced into said cells as a lipid complex comprising a cationic lipid suitable for transfecting cells.

26. The method of any one of claims 16-18 and 21-22, wherein the target sequence encodes an antisense RNA molecule.

27. The method of any one of claims 16-18 and 21-22, wherein said target sequence encodes a protein or polypeptide.

28. A cell line stably transformed with the autoregulatory sequence of claim 1.

29. A method of expressing a target sequence in the stably transformed cell line of claim 28, said method comprising introducing into said cell line nucleic acid sequence comprising a prokaryotic promoter operatively linked to the target sequence, wherein transcription of said target sequence is initiated by the RNA polymerase expressed in the stably transformed cell line.

30. A pharmaceutical composition comprising the autoregulatory sequence according to any one of claims 9-11.

31. A pharmaceutical composition comprising a first nucleic acid sequence having a eukaryotic promoter operatively linked to a prokaryotic promoter which is operatively linked to a nucleic acid sequence encoding a prokaryotic promoter which initiates transcription,

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through said prokaryotic promoter, of the sequence encoding said RNA polymerase; and

a second nucleic acid sequence comprising a second copy of the prokaryotic promoter operatively linked to a target acid sequence.

32. A pharmaceutical composition comprising a single sequence having a target sequence operatively linked to the prokaryotic promoter of the autoregulatory sequence of claim 1.

33. The pharmaceutical composition of claims 30-32, wherein the sequences are present in a lipid complex comprising a cationic lipid suitable for transfecting cells.

34. A method for expressing a linear double-stranded target sequence in cells; said method comprising: complexing said target sequence with cationic liposomes and introducing said complex to said cells.

35. A method for expressing a linear double-stranded target sequence in cells, said method comprising: complexing said target sequence with polycation and cationic liposomes and introducing said complex to said cells.

36. The method of claims 34 or 35, wherein the target sequence is operatively linked to a eukaryotic promoter.

37. The method of claim 34, wherein said target sequence is chemically synthesized.

38. The method of claim 35, wherein said target sequence is chemically synthesized.

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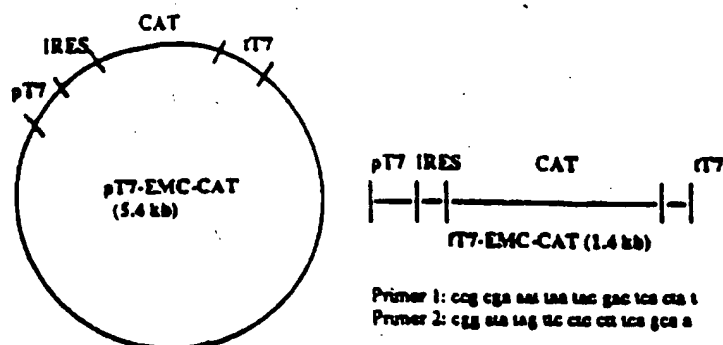
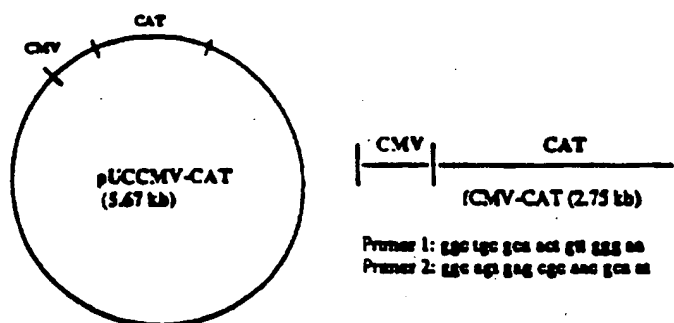
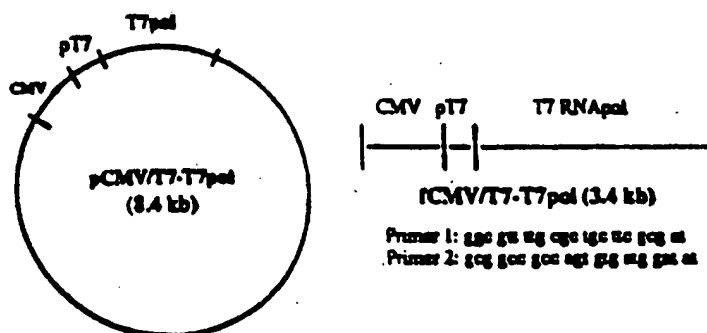
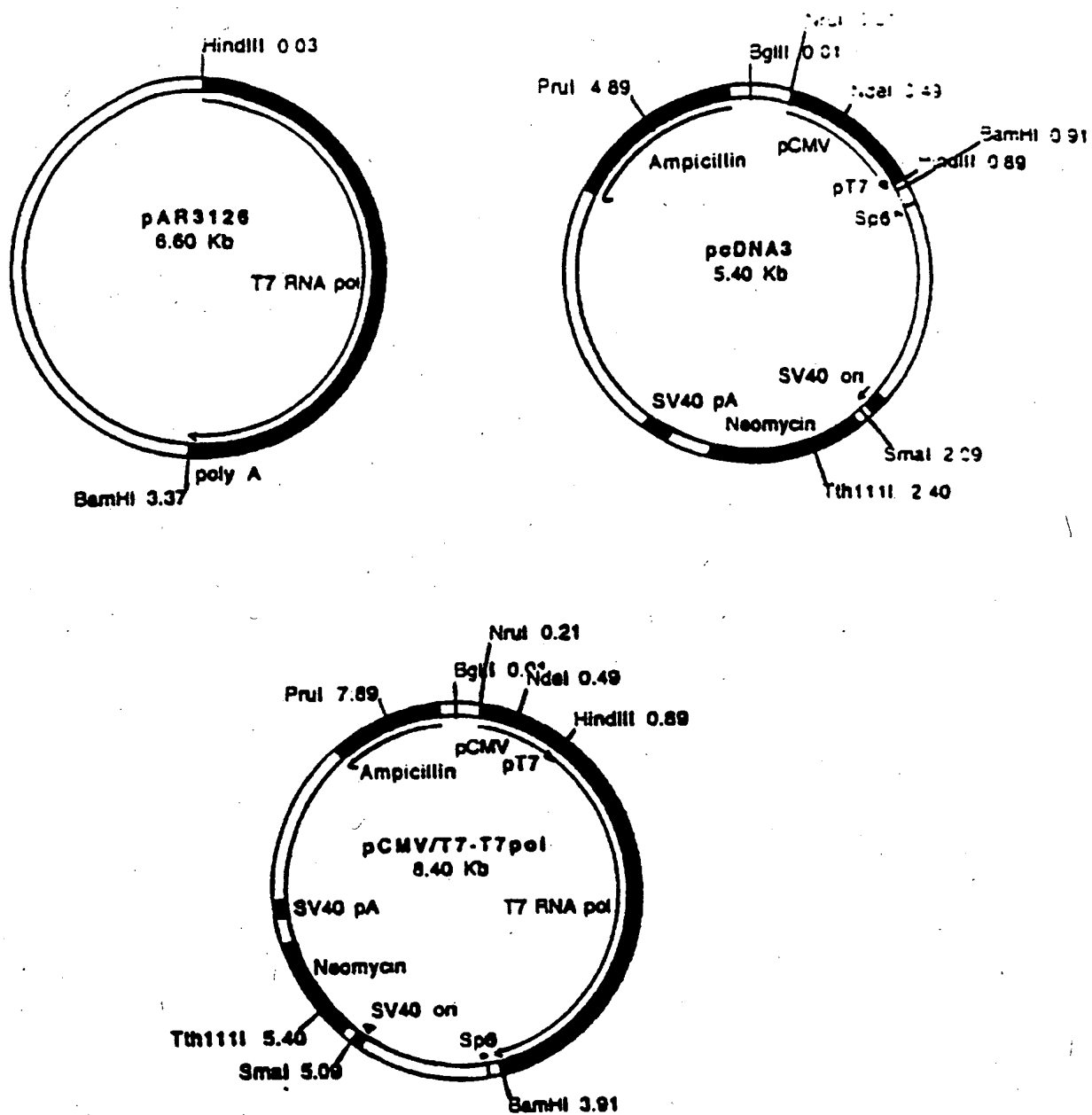
**FIGURE 1****Cytoplasmic expression system****Nuclear expression system****Dual expression system**

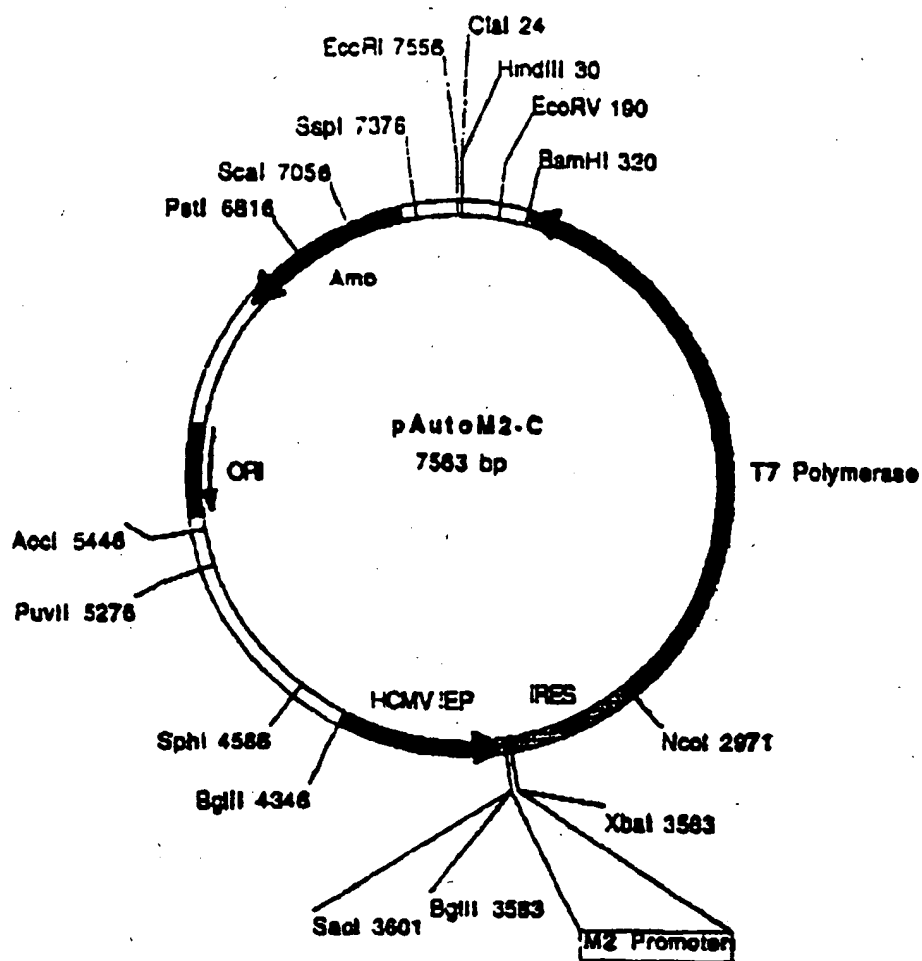
FIGURE 2





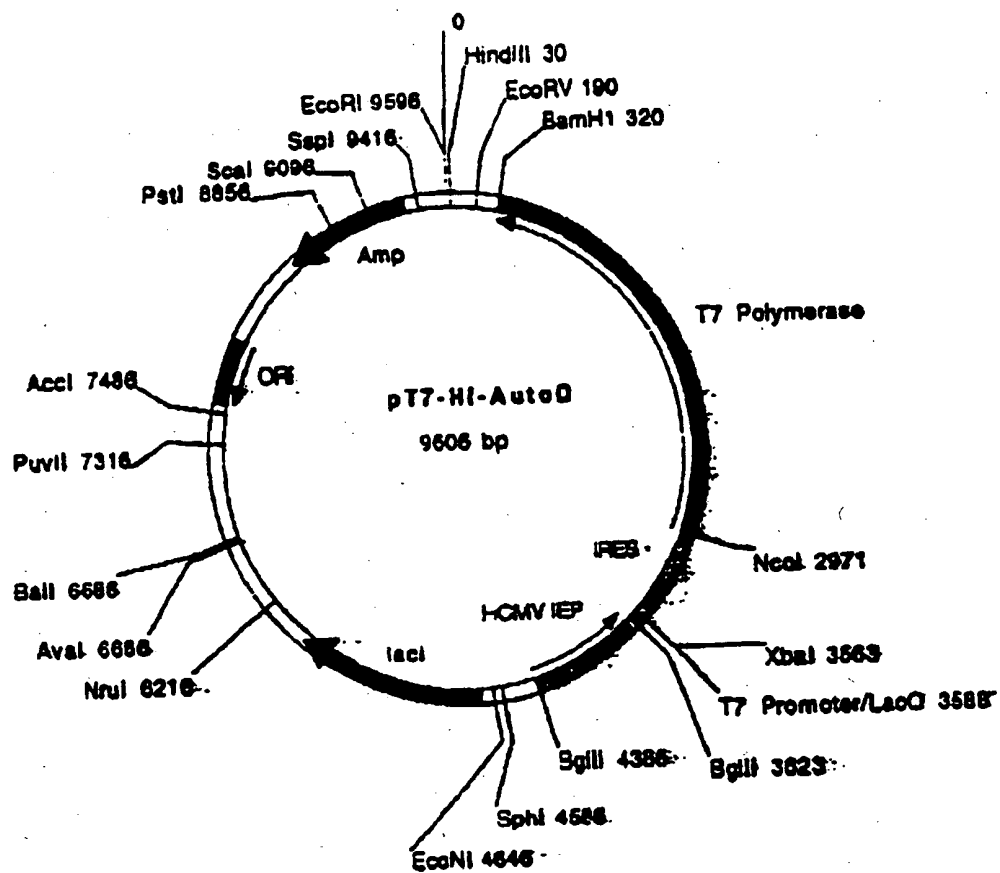
3/30

FIGURE 3

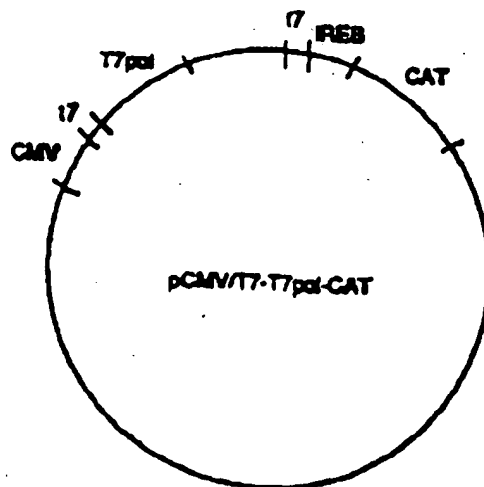


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FIGURE 4



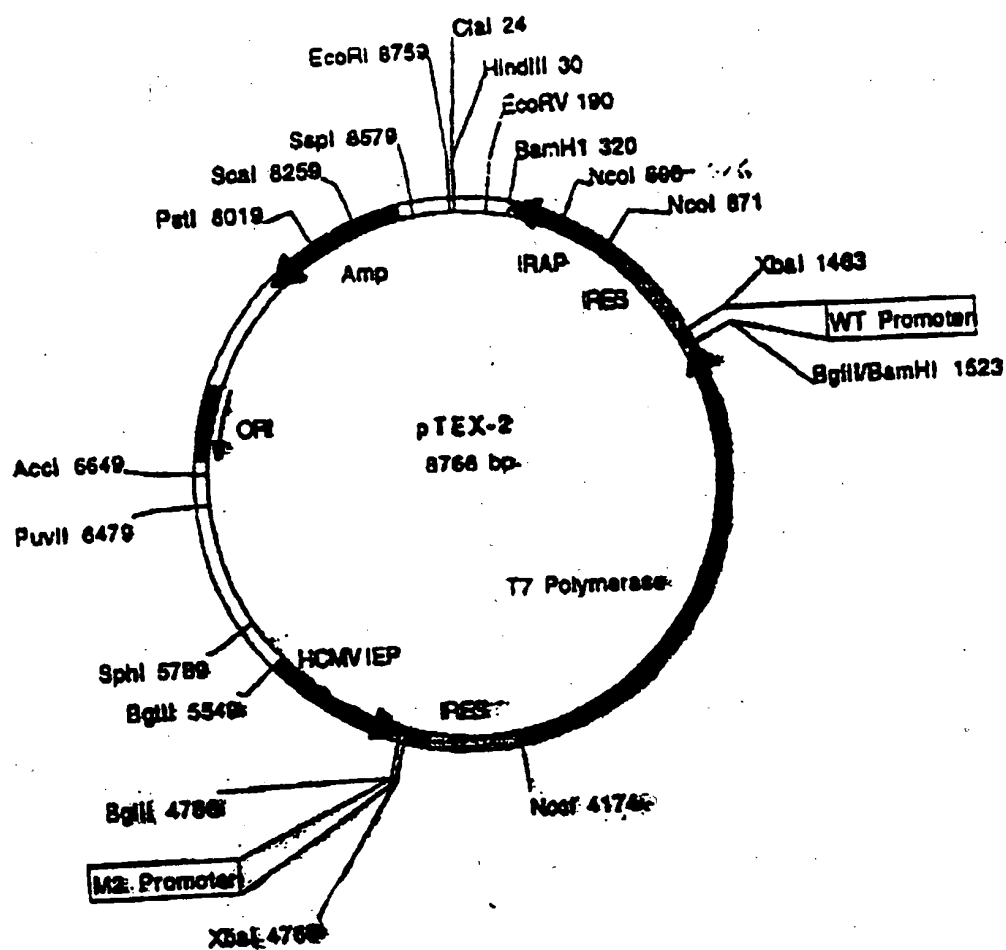
5/30

**FIGURE 5**

A dual expression plasmid capable of both self-amplification of T7 RNA polymerase and the expression of reporter gene driven by the T7 promoter.

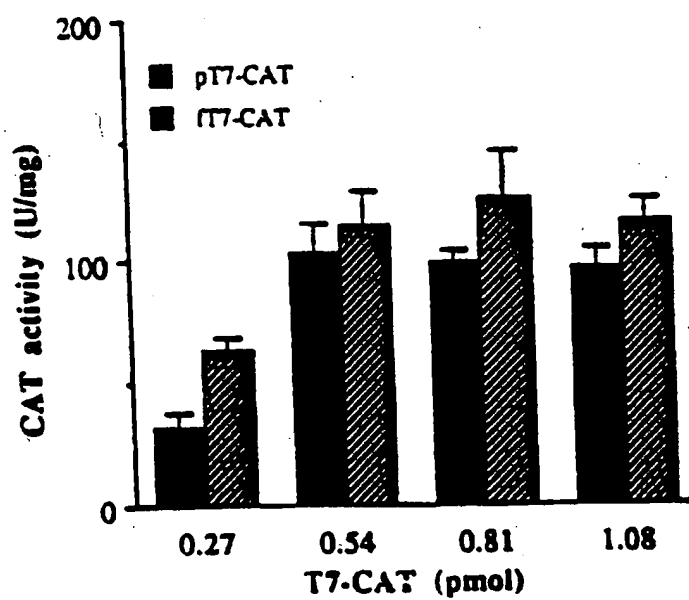
6/30

FIGURE 6



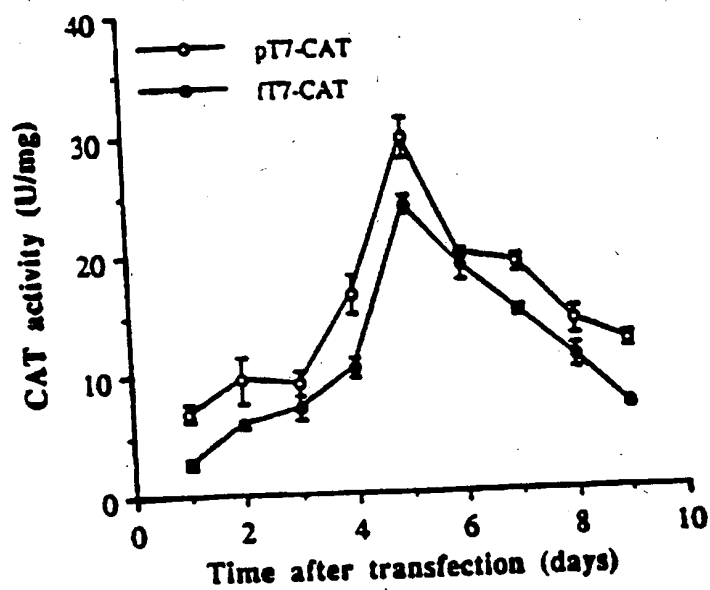
7/30

FIGURE 7



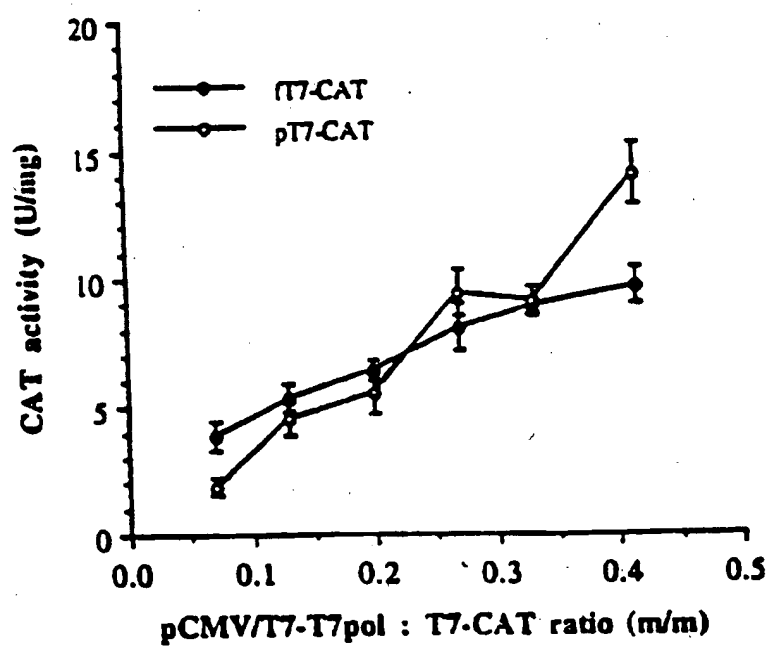
8/30

FIGURE 8



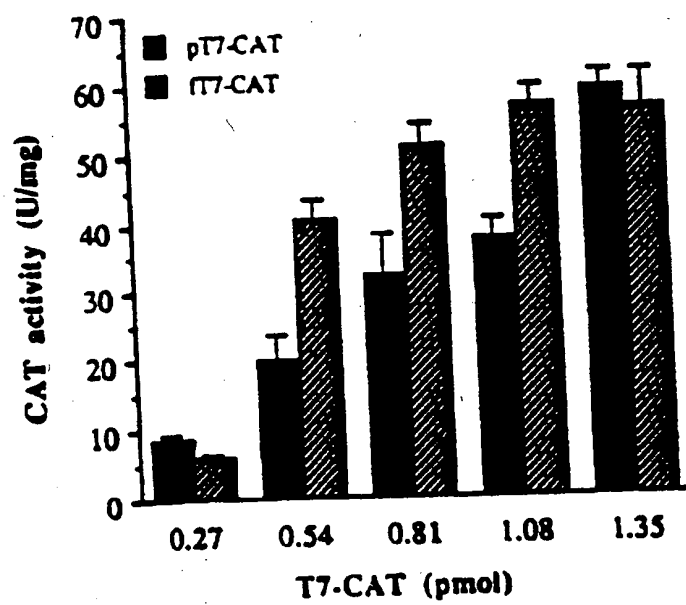
9/30

FIGURE 9



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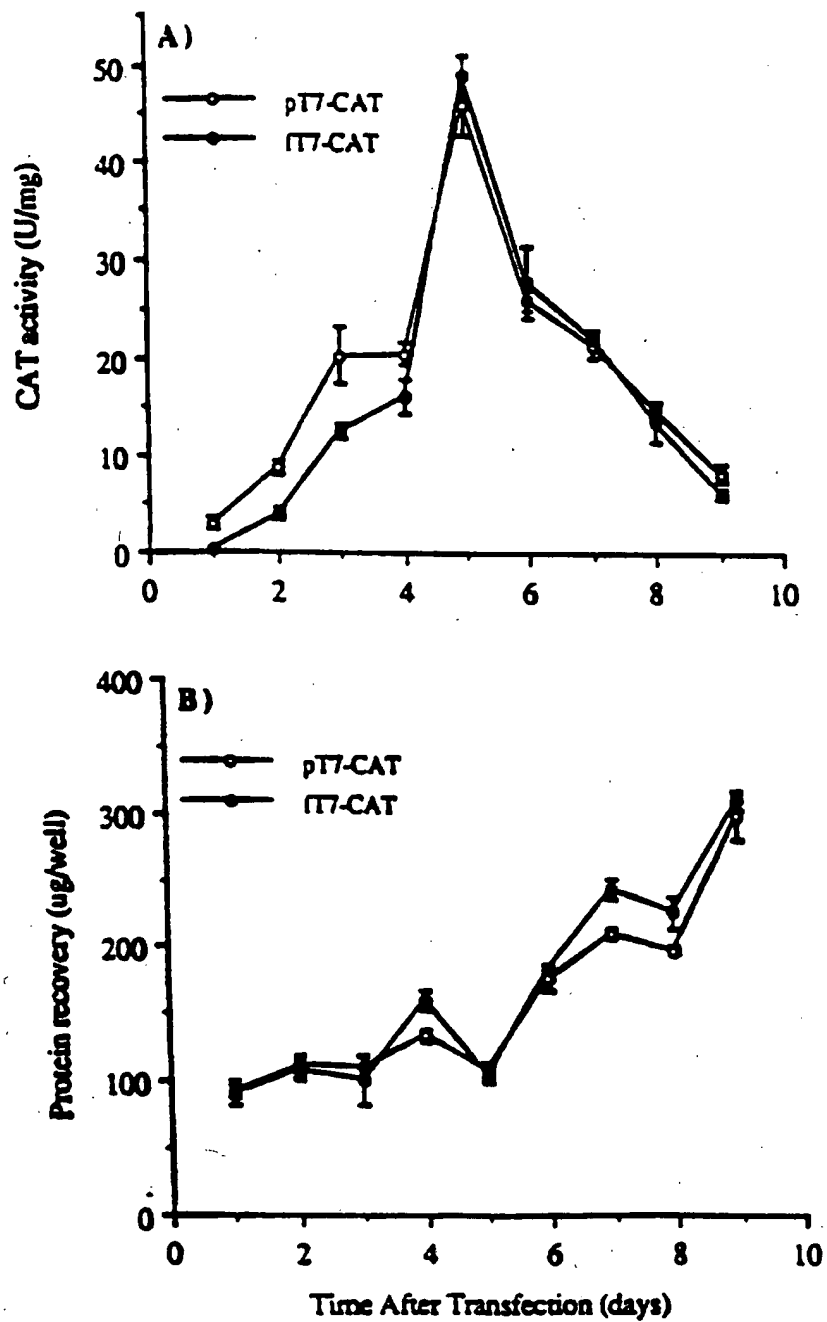
FIGURE 10





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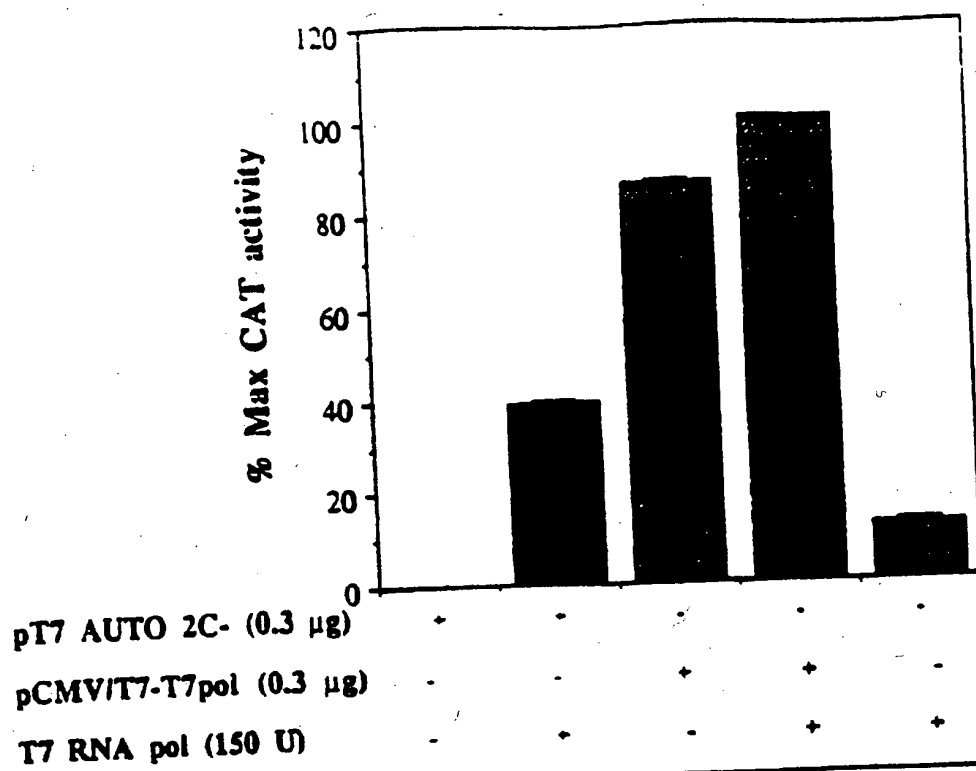
FIGURE 11



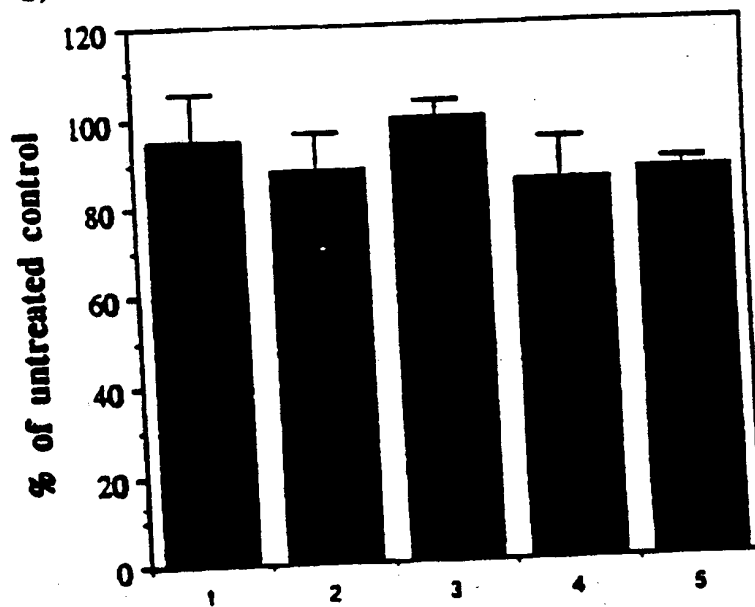
12/30

FIGURE 12

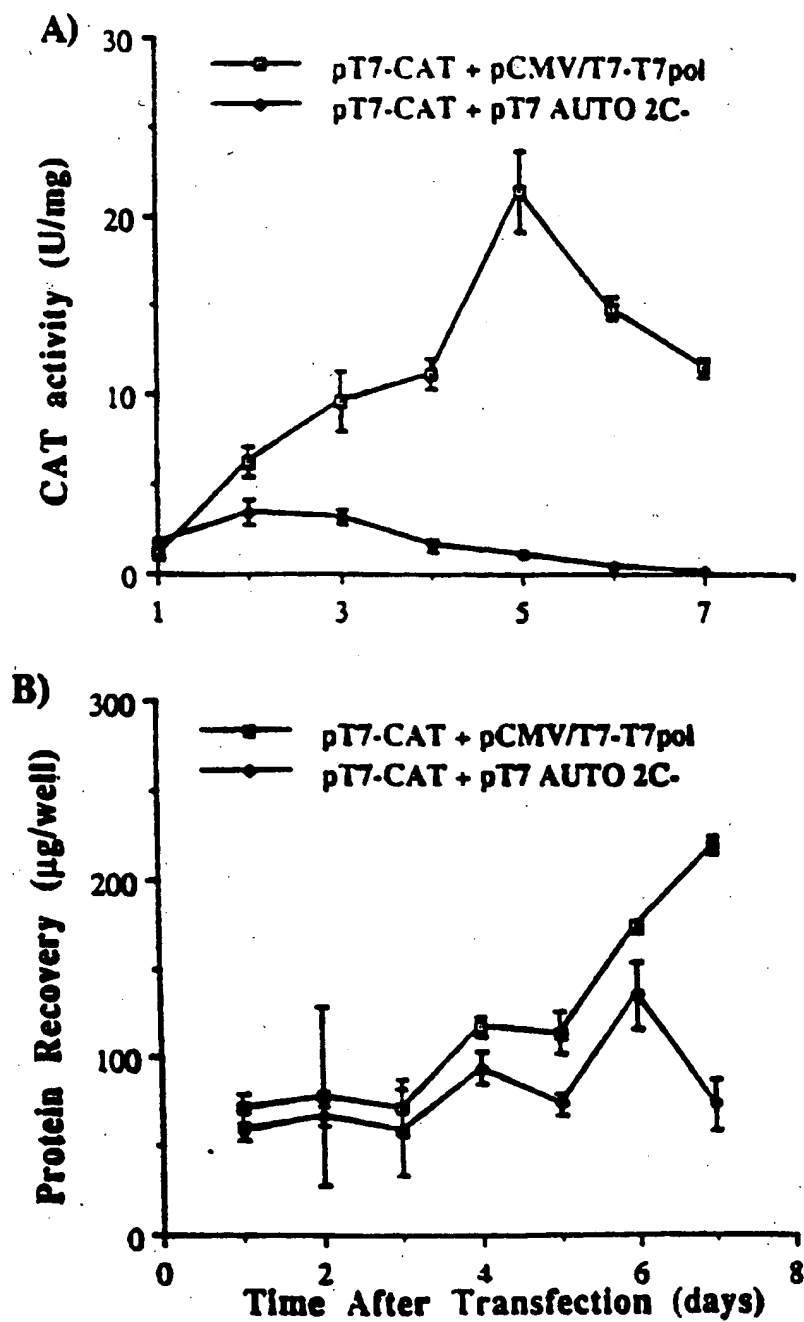
A)



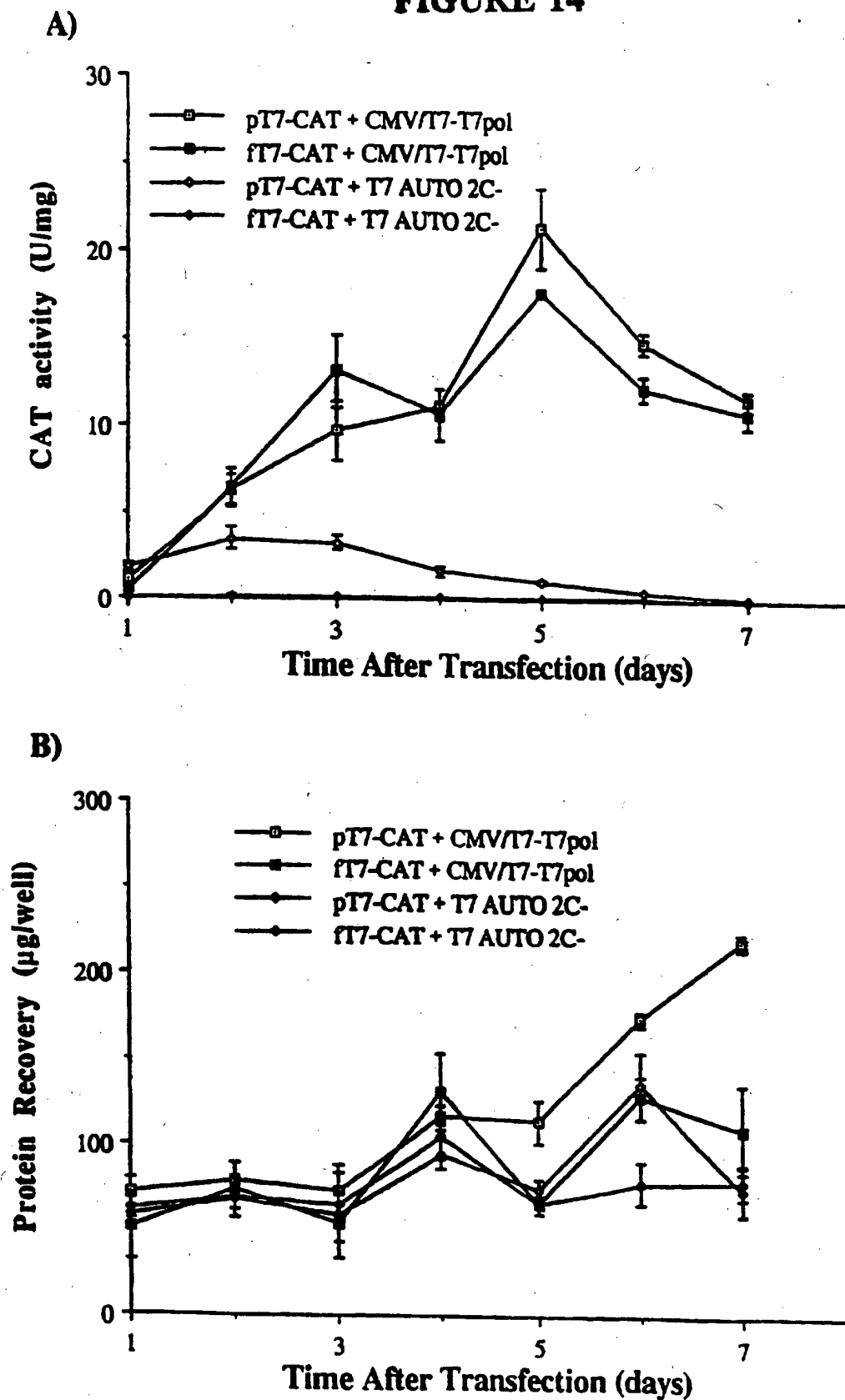
B)



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**FIGURE 13**

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**FIGURE 14**

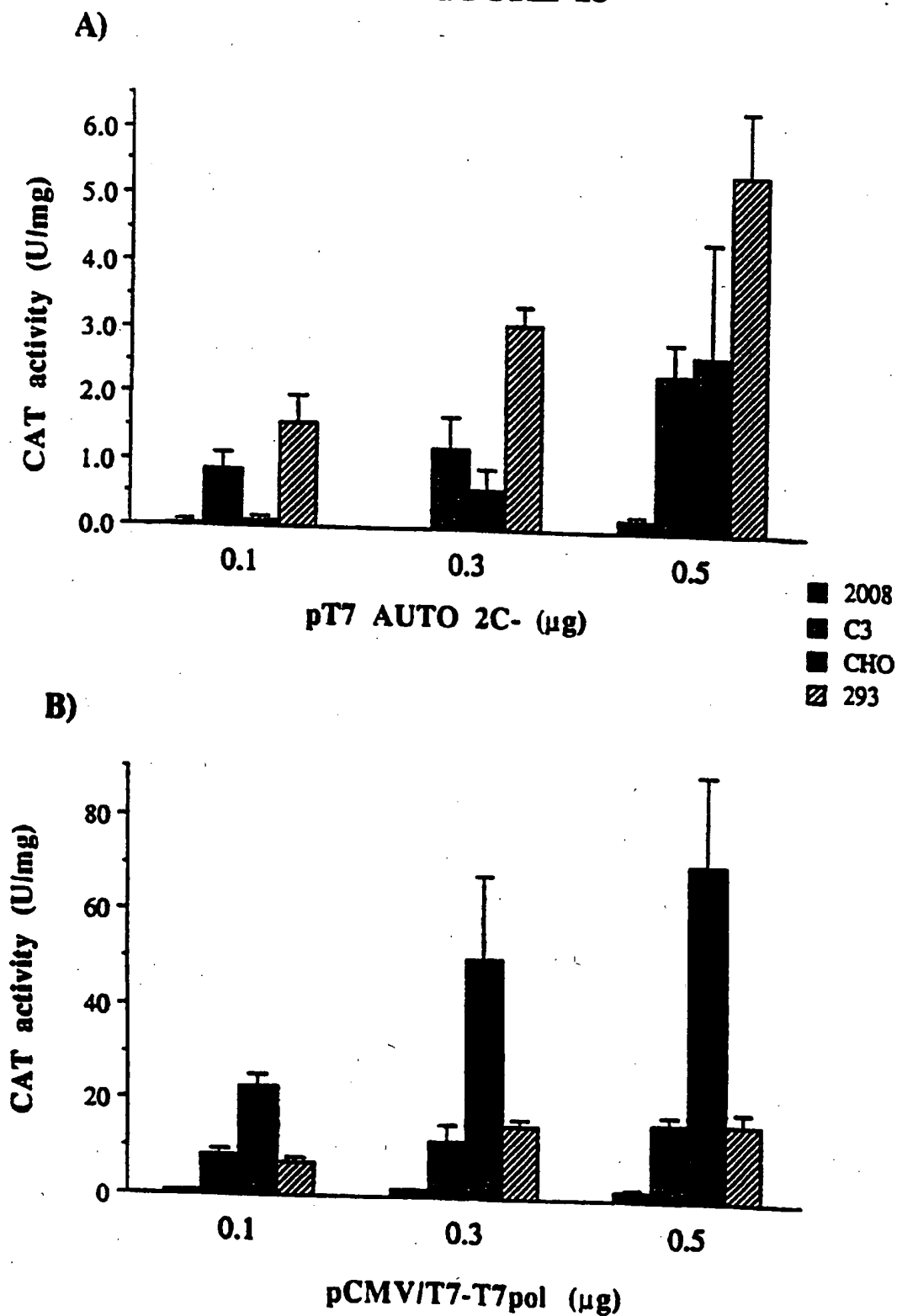
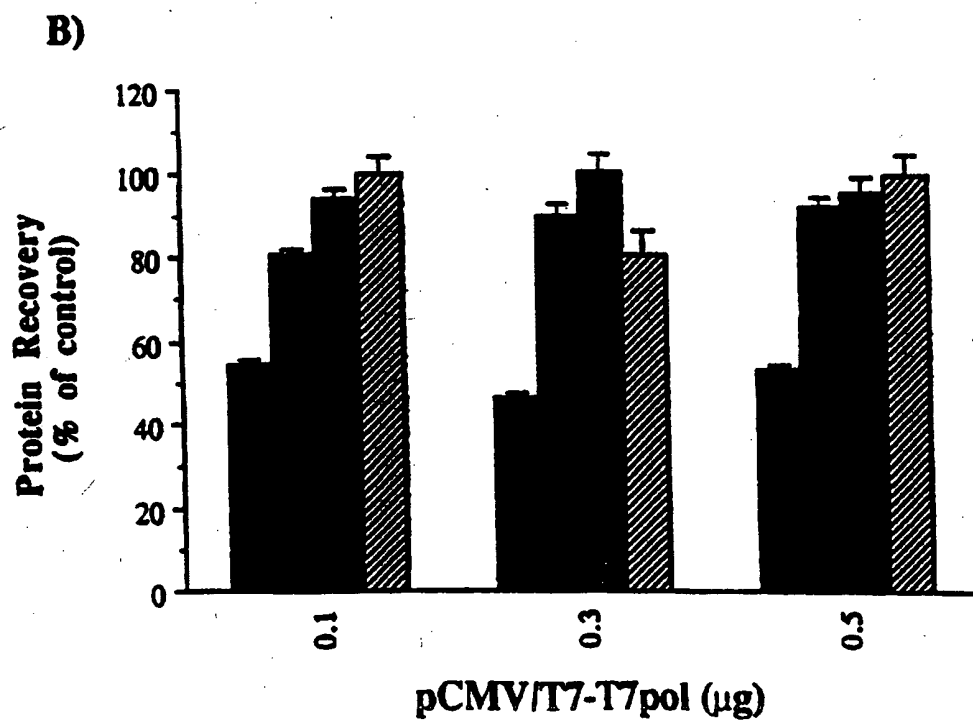
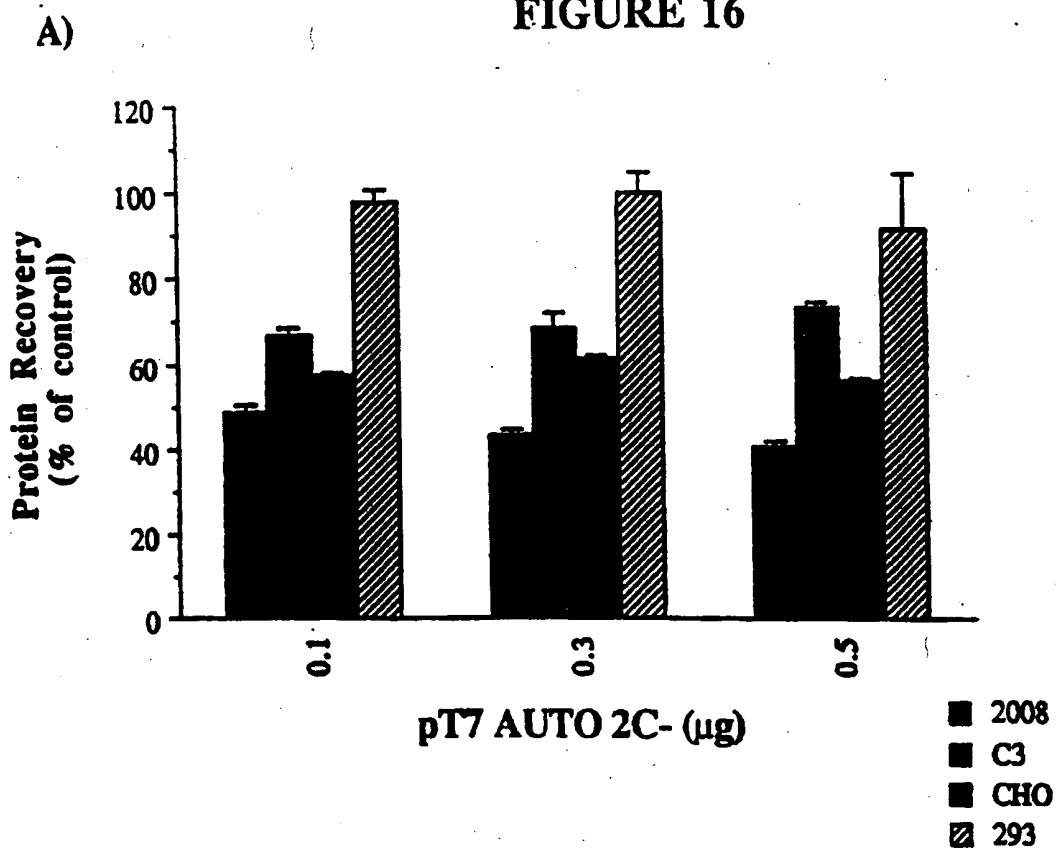
**FIGURE 15**

FIGURE 16



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FIGURE 17

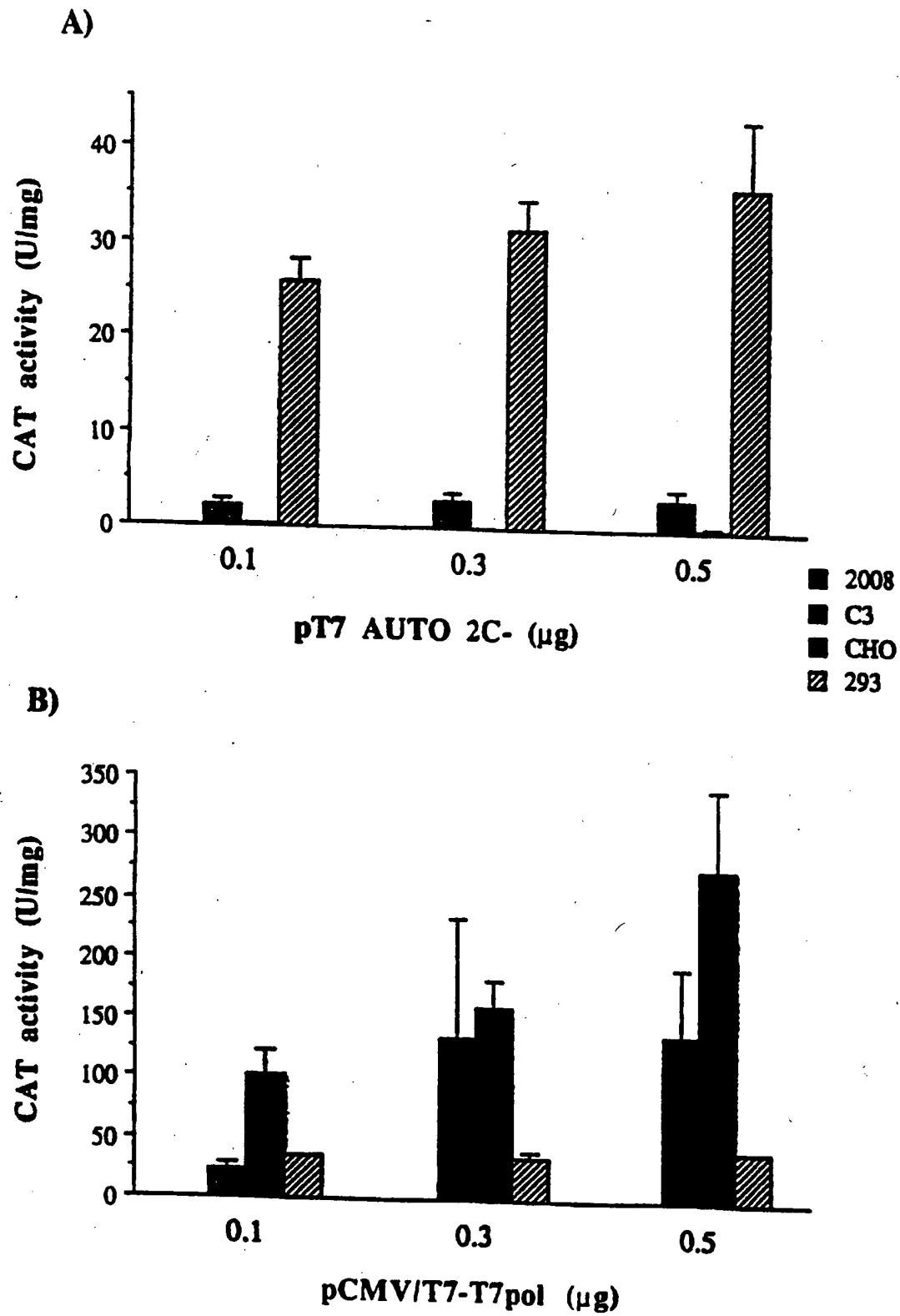
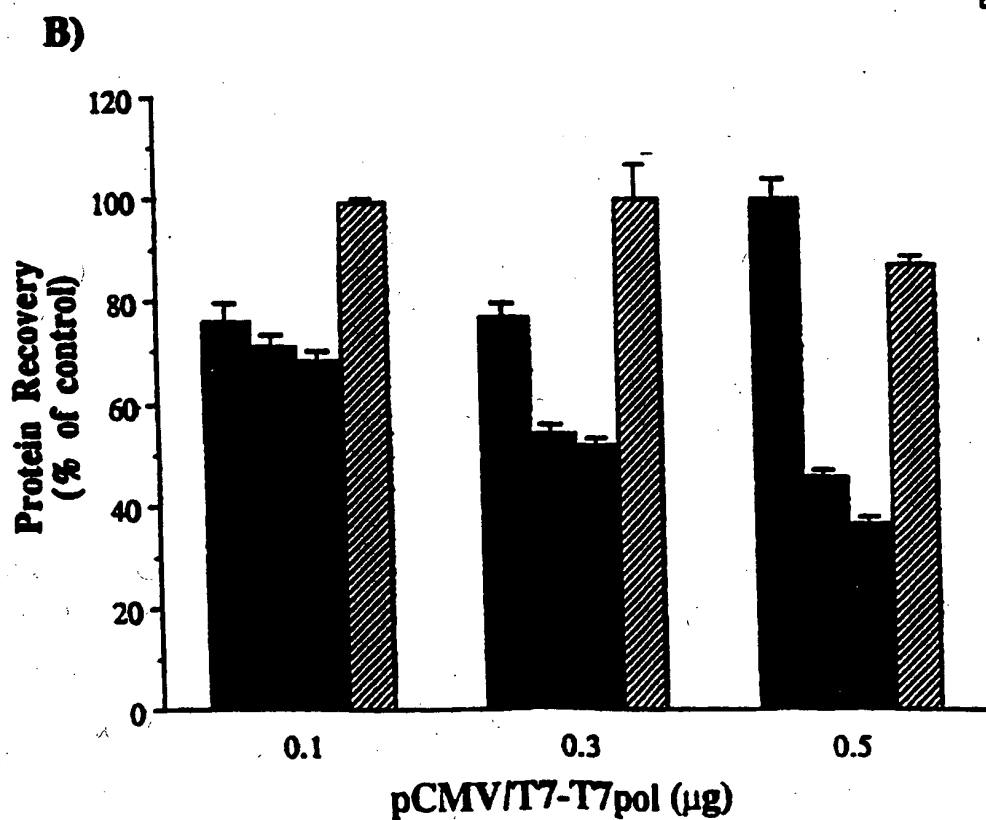
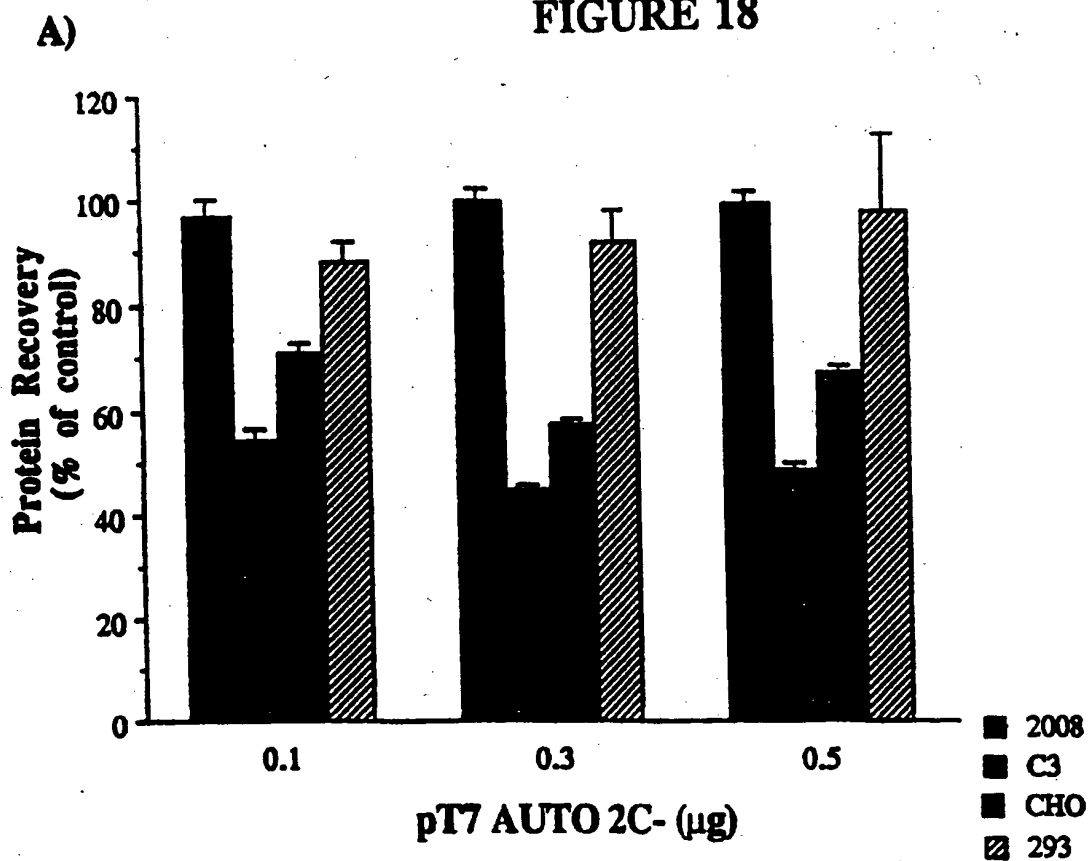


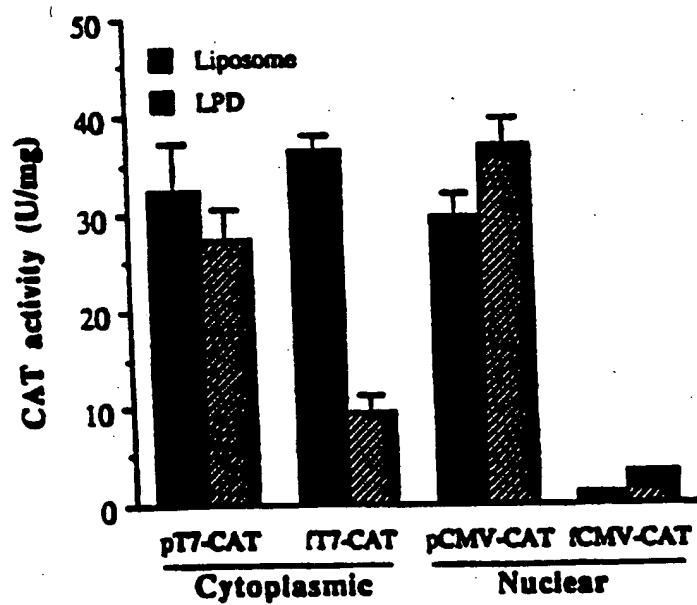
FIGURE 18





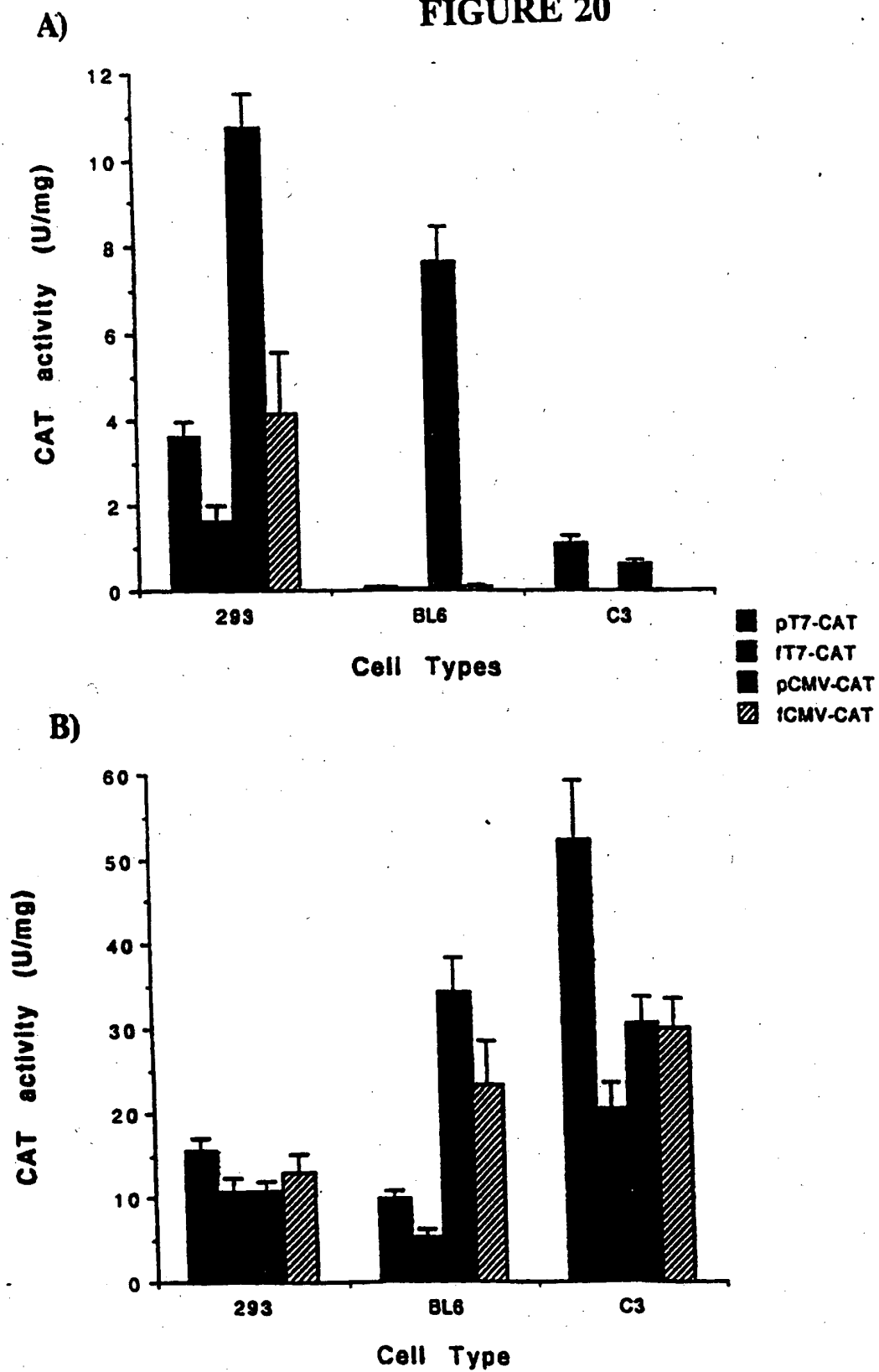
19/30

FIGURE 19



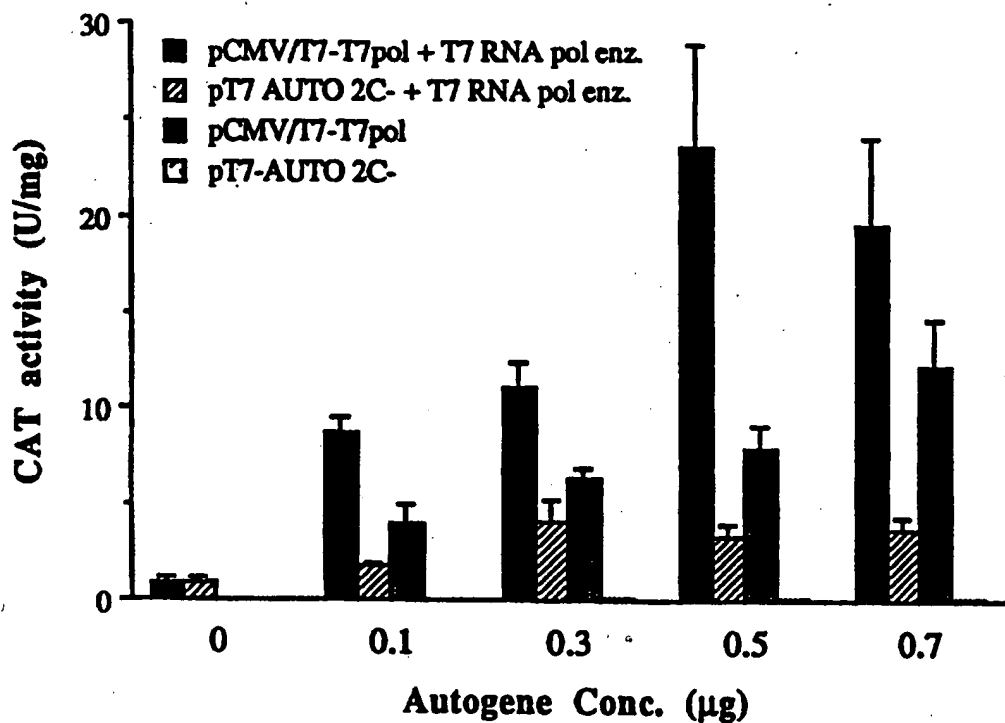
20/30

FIGURE 20



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21 A



21 B

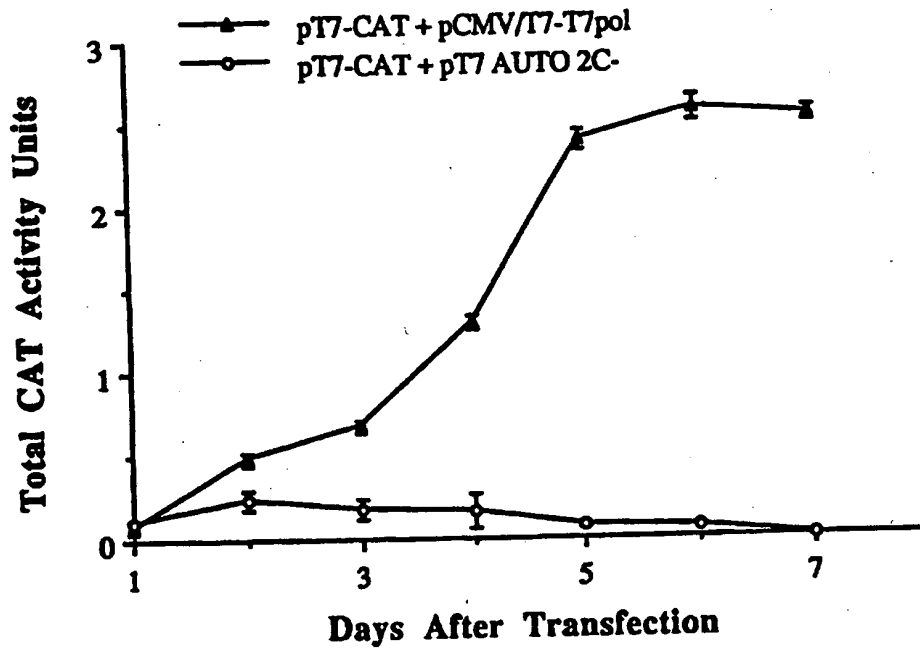
|                | a | b | c | d | e | f |
|----------------|---|---|---|---|---|---|
| pCMV/T7-T7pol  | - | + | + | - | - | - |
| pT7 AUTO 2C-   | - | - | - | + | + | + |
| T7 RNA pol enz | - | - | + | - | - | + |



Figures 21A, 21B

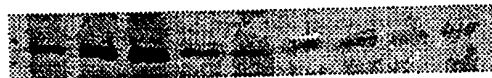
22/30

22 A



22 B

|                        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------------|---|---|---|---|---|---|---|---|---|
| pCMV/T7-T7pol          | + | + | + | + | - | - | - | - | - |
| pT7 AUTO 2C- + enz     | - | - | - | - | + | + | + | + | - |
| Day after transfection | 1 | 3 | 5 | 7 | 1 | 3 | 5 | 7 | - |

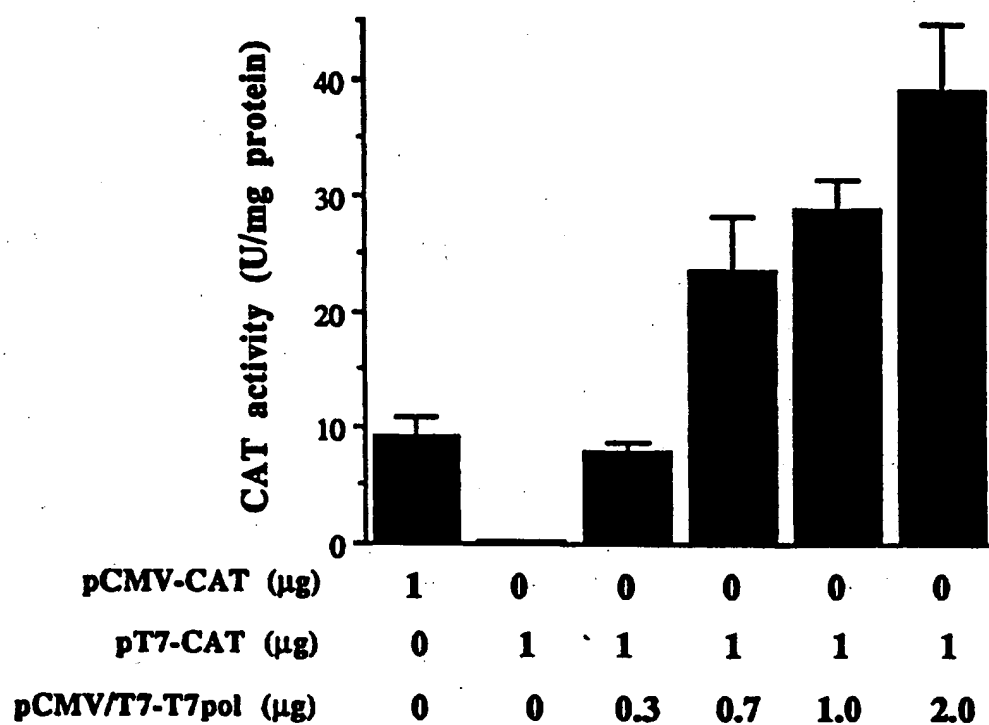


Total T7 RNA polymerase (relative units)

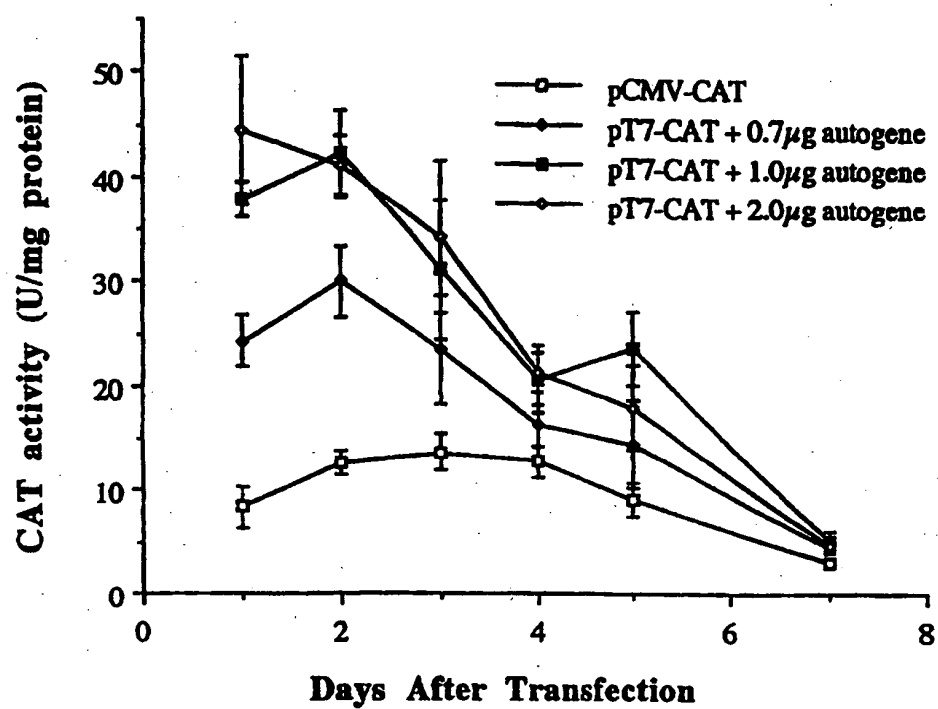
|  |    |     |    |    |   |   |   |   |   |
|--|----|-----|----|----|---|---|---|---|---|
|  | 11 | 116 | 91 | 31 | - | - | - | - | - |
|--|----|-----|----|----|---|---|---|---|---|

Figures 22A, 22B

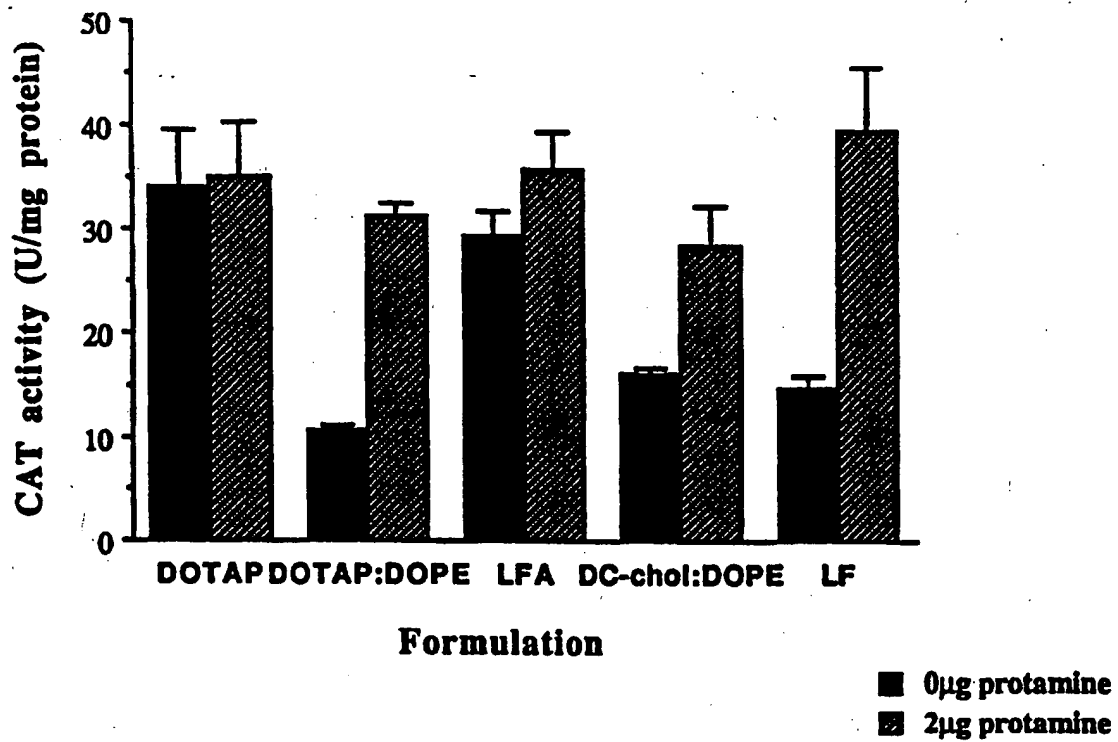
23/30

*Figure 23*

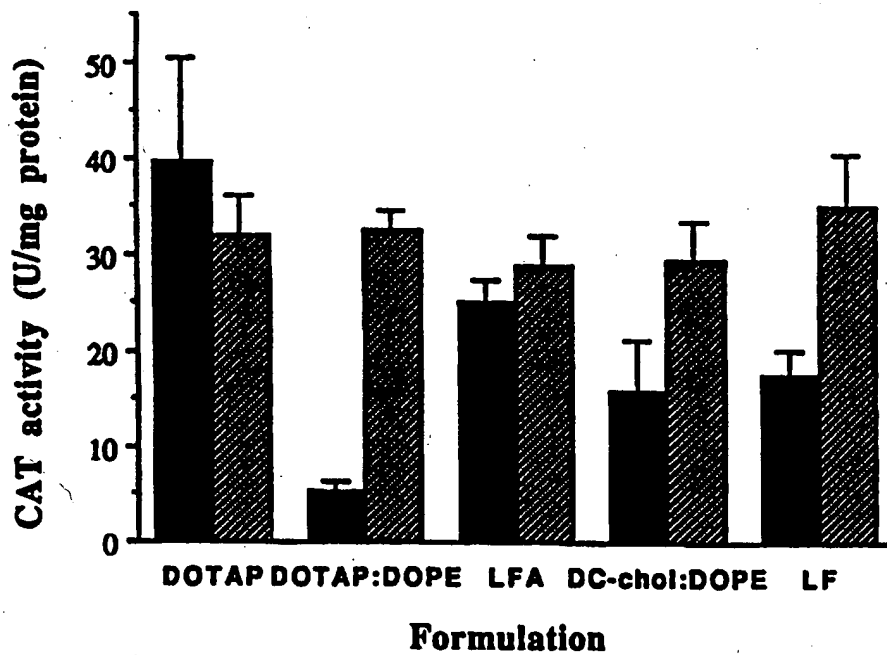
24/30

*Figure 24*

25 A



25 B

*Figures 25A, 25B*

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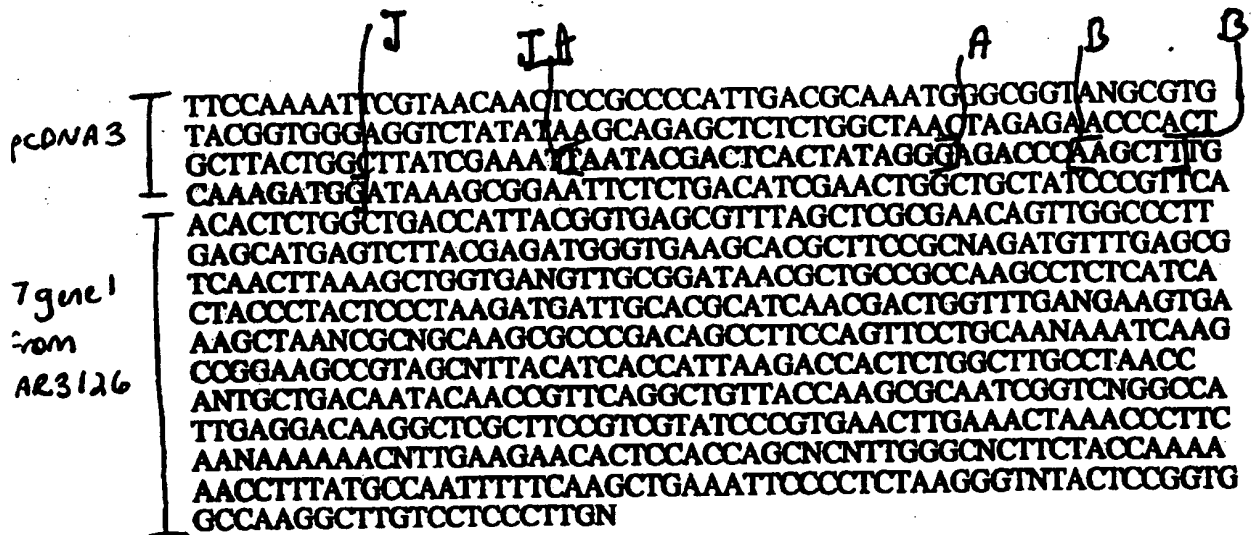


Figure 26



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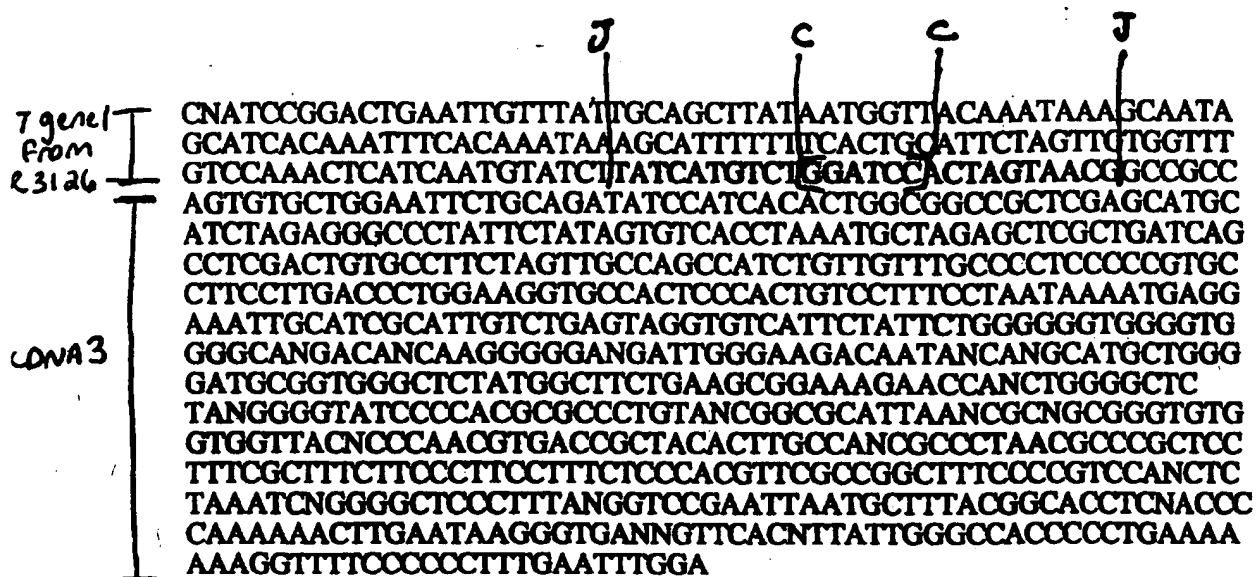


Figure 27

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*Hind*III

1 AAGCTTTGCA AAGATGGATA AAGCGGAATT CTCTGACATC  
GAACTGGCTG CTATCCCGTT  
61 CAACACTCTG GCTGACCATT ACGGTGAGCG TTTAGCTCGC  
GAACAGTTGG CCCITGAGCA  
121 TGAGTCTTAC GAGATGGGTG AAGCACGCTT CCGCAAGATG  
TTTGAGCGTC AACTTAAAGC  
181 TGGTGAGGTT GCGGATAACG CTGCCGCCAA GCCTCTCATC ACTACCCTAC  
TCCCTAAGAT  
241 GATTGCACGC ATCAACGACT GGTTTGAGGA AGTGAAAGCT  
AAGCGGGCA AGCGCCCGAC  
301 AGCCTTCCAG TTCCTGCAAG AAATCAAGCC GGAAGCCGTA  
GCGTACATCA CCATTAAGAC  
361 CACTCTGGCT TGCCTAACCA GTGCTGACAA TACAACCGTT  
CAGGCTGTAG CAAGCGCAAT  
421 CGGTCGGGCC ATTGAGGACG AGGCTCGCTT CGGTCGTATC CGTGACCTTG  
AAGCTAAGCA  
481 CTTCAAGAAA AACGTTGAGG AACAACTCAA CAAGCGCGTA  
GGGCACGTCT ACAAGAAAGC  
541 ATTTATGCAA GTTGTGCGAGG CTGACATGCT CTCTAAGGGT  
CTACTGGGTG GCGAGGCGTG  
601 GTCTTCGTGG CATAAGGAAG ACTCTATTCA TGTAGGAGTA  
CGCTGCATCG AGATGCTCAT  
661 TGAGTCAACC GGAATGGTTA GCTTACACCG CCAAATGCT  
GGCGTAGTAG GTCAAGACTC  
721 TGAGACTATC GAACTCGCAC CTGAATACGC TGAGGCTATC  
GCAACCGGTG CAGGTGCGCT  
781 GGCTGGCATC TCTCCGATGT TCCAACCTTG CGTAGTTCCT CCTAAGCOGT  
GGACTGGCAT  
841 TACTGGTGGT GGCTATTGGG CTAACGGTCG TCGTCCTCTG GCGCTGGTGC  
GTACTCACAG  
901 TAAGAAAGCA CTGATGCGCT ACGAAGACGT TTACATGCCT  
GAGGTGTACA AAGCGATTAA  
961 CATTGCGCAA AACACCGCAT GGAAAATCAA CAAGAAAGTC  
CTAGCGGTG CCAACGTAAT  
1021 CACCAAGTGG AAGCATTGTC CGGTCGAGGA CATCCCTGCG  
ATTGAGCGTG AAGAACTCC  
1081 GATGAAACCG GAAGACATCG ACATGAATCC TGAGGCTCTC  
ACCGCGTGGA AACGTGCTGC  
1141 CGCTGCTGTG TACCGCAAGG ACAAGGCTCG CAAGTCTCGC CGTATCAGCC  
TTGAGTTCAT

*Figure 28*

1201 GCTTGAGCAA GCCAATAAGT TTGCTAACCA TAAGGCCATC  
TGGTTCCTT ACAACATGGA  
1261 CTGGCGCGGT CGTGTTTACG CTGTGTCAAT GTTCAACCCG  
CAAGGTAACG ATATGACCAA  
1321 AGGACTGCTT ACGCTGGCGA AAGGTA AACC AATCGGTAAG  
GAAGGTTACT ACTGGCTGAA  
1381 AATCCACGGT GCAAACGTG CGGGTGTCGA TAAGGTTCCG  
TTCCCTGAGC GCATCAAGTT  
1441 CATTGAGGAA AACCACGAGA ACATCATGGC TTGCGCTAAG  
TCTCCACTGG AGAACACTTG  
1501 GTGGGCTGAG CAAGATTCTC CGTCTGCTT CCTTGC GTTC TGCTTTGAGT  
ACGCTGGGGT  
1561 ACAGCACCAC GGCCTGAGCT ATA ACTGCTC CCTTCCGCTG GCGTTTGACG  
GGTCTTGCTC  
1621 TGGCATCCAG CACTTCTCCG CGATGCTCCG AGATGAGGTA  
GGTGGTGGG CGGTAACTT  
1681 GCTTCCTAGT GAAACCGTTC AGGACATCTA CGGGATTGTT  
GCTAAGAAAG TCAACGAGAT  
1741 TCTACAAGCA GACGCAATCA ATGGGACCGA TAACGAAGTA  
GTTACCGTGA CCGATGAGAA  
1801 CACTGGTGAA ATCTCTGAGA AAGTCAAGCT GGGCACTAAG  
GCACTGGCTG GTCAATGGCT  
1861 GGCTTACGGT GTTACTCGCA GTGTGACTAA GCGTTCAGTC  
ATGAOGCTGG CTTAOGGGTC  
1921 CAAAGAGTTC GGCTTCCGTC AACAAGTGCT GGAAGATACC  
ATTAGCCAG CTATTGATTC  
1981 CGGCAAGGGT CTGATGTTCA CTCAGCCGAA TCAGGCTGCT  
GGATACATGG CTAAGCTGAT  
2041 TTGGGAATCT GTGAGCGTGA CCGTGGTAGC TGCGGTTGAA  
GCAATGAACT GGCTTAAGTC  
2101 TGCTGCTAAG CTGCTGGCTG CTGAGGTCAA AGATAAGAAG  
ACTGGAGAGA TTCTTCGCAA  
2161 GCGTTGCGCT GTGCATTGGG TAACTCCTGA TGGTTTCCCT GTGTGGCAGG  
AATACAAGAA  
2221 GCCTATTAG ACGCGCTTGA ACCTGATGTT CCTCGGTCAG TTCCGCTTAC  
AGCCTACCAT  
2281 TAACACCAAC AAAGATAGCG AGATTGATGC ACACAAACAG  
GAGTCTGGTA TCGCTCCTAA  
2341 CTTTGTACAC AGCCAAGACG GTAGCCACCT TCGTAAGACT  
GTAGTGTGGG CACACGAGAA  
2401 GTACGGAATC GAATCTTTTG CACTGATTCA CGACTCCTTC  
GGTACCATTCCGGCTGACGC

*Figure 28 cont*

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2461 TCGGAACCTG TTCAAAGCAG TCGCGGAAAC TATGGTTGAC  
ACATATGAGT CTTGTGATGT  
2521 ACTGGCTGAT TTCTACGACC AGTTCGCTGA CCAGTTGCAC  
GAGTCTCAAT TGGACAAAAT  
2581 GCCAGCACTT CCGGCTAAAG GTAAC TTGAA CCTCCGTGAC  
ATCTTAGAGT CGGACTTCGC  
2641 GTTCGCGTAA CGCCAAATCA ATACGACTCC GGATCTCGAA  
CTTGTTTATT GCAGCTTATA  
2701 ATGGTTACAA ATAAAGCAAT AGCATCACAA ATTCACAAA  
TAAAGCATT TTTTCACTGC  
2761 ATTCTAGTTG TGGTTTGTCC AAAC TCATCA ATGTATCTTA  
TCATGTCGGATCC  
BAMHI

*Figure 28 cont.*